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Standing Committee for the European Medical Research Councils (EMRC)

ESF Exploratory Workshop on

BioBor – Exploring New Opportunities of Boron Chemistry Towards Medicine

Lodz, Poland, May 9 – 12, 2008

Convened by:
Zbigniew J. Lesnikowski and Agnieszka Olejniczak

Institute of Medical Biology, Polish Academy of Sciences, Lodz

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Main Objectives of the Workshop:

The chemistry of boron has many aspects and a long history. Boron containing compounds find broad use in many areas of research and practice. The important stimulus for development of bioorganic and medicinal chemistry of boron was provided by revival of interests in boron neutron capture therapy (BNCT) of cancers. Albeit, the knowledge accumulated during past decades on chemistry and biology of bioorganic boron derivatives laid at the turn of XX and XXI century a foundation for new areas of study and application of boron compounds and calls for new ideas.

The application of boron clusters as lipophilic pharmacophores and modulators of biologically active molecules is one of several examples. In this field boron clusters play an important role. Simultaneously new, previously unknown biological activities of boron cage molecules have been discovered, they include anti-HIV activity, anticancer activity or in the form of conjugates with nucleosides (nucleic acid components) activity as potential receptors modulators, and others.

The aim of the workshop is to bring together scientists working on other aspects of bioorganic chemistry of boron than BNCT and to discuss and define fresh potentialities offered by recent discoveries, often done in participants' laboratories. The BNCT will not be excluded but it will not be the main topic of the meeting. This should give rise to new concepts and help to consolidate research programs of participants whenever possible and profitable. Many prospective participants of the meeting are already involved in collaborative programs, it is expected however that a frame for a broader joint program will be created during the workshop.

Convenors:

Zbigniew J. Lesnikowski
Agnieszka Olejniczak

PROGRAMME

Individual presentations will be 35 minutes followed by 10 minutes discussion.

Friday 9 May 2008

Afternoon/Evening	*Arrival and registration at "Qubus" hotel*

Saturday 10 May 2008

Session 1

09:00-09:30	**Meeting introduction by the convenors** **Alexandra Polakova** (Standing Committee for the European Medical Research Councils) **Presentation of the European Science Foundation (ESF)**
09:30-10:15	**Vladimir I. Bregadze** (INEOS) Synthesis of new polyhedral boron compounds as potential antitumour agents
10:15-11:00	**Petr Cigler** (VSCHT) Fluorescently labeled metallacarboranes: solution behavior and interaction with serum proteins
11:00-11:15	*Coffee break*
11:15-12:00	**Staffan Eriksson** (SUAS) DNA precursor enzymes and carborano nucleosides
12:00-12:45	**Bohumir Grüner** (IIC) Metallacarborane building blocks and their use in design of inhibitors of HIV protease
12:45-13:30	**M. Frederic Hawthorne** (IINMM) Roles for polyhedral boranes and carboranes in nano and molecular medicine

13:30-14:45 *Lunch*

Session 2

14:45-15:30 **Evamarie Hey-Hawkins** (Universität Leipzig)
Imitation and Modification of Biologically Relevant or Active Molecules via Integration of Carbaborane Clusters

15:30-15:45 *Coffee break*

15:45-16:30 **Jan Konvalinka** (IOCB)
Metallacarboranes as potent and specific inhibitors of HIV protease and its resistant variants: more inhibitors, more enzymes, more structures

16:30-17:15 **Zbigniew Lesnikowski** (IBM PAS)
Beyond pyrimidine nucleosides and carboranes - New nucleoside/boron cluster conjugates and their applications

18:00-21:00 *Workshop Dinner* and Bar Discussion

Sunday 11 May, 2008

Session 3

09:30-10:15 **Pavel Matějíček** (Charles University)
Behavior of metallacarboranes in aqueous solutions and their interaction with polymers

10:15-11:00 **Hiroyuki Nakamura** (Gakushuin University)
Boronic acid as an alternative functional group for drug design

11:00-11:15 *Coffee break*

11:15-12:00 **Agnieszka Olejniczak** (IBM PAS)
Boron clusters as electrochemical labels for biomolecules

12:00-12:45 **Michael D. Threadgill** (University of Bath)
1,2-Dicarbadodecaboranes: chemical strategies fordelivery and opportunities as ligand platforms

12:45-13:30 **Werner Tjarks** (Ohio State University)
The carborane cluster in computational drug design

13:30-15:00	*Lunch*
	Session 4
15:00-15:45	**Clara Viñas** (ICMAB) Carboranes and metallacarboranes as building blocks for biomaterials
15:45-16:30	**Marek Zaidlewicz** (Nicolaus Copernicus University) Asymmetric synthesis of 5-lipoxygenase Inhibitors
16:30-16:45	*Coffee break*
16:45-18:15	**Brainstorming and plans for action**
18:15-20:30	*Dinner* and Bar Discussion

Monday 12 May 2008

08:00	*Breakfast and departure*

List of Participants

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Contents

SYNTHESIS OF NEW POLYHEDRAL BORON COMPOUNDS AS POTENTIAL ANTITUMOR AGENTS

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Summary

Currently polyhedral boron compounds are likely to possess a wide range of possible application in the field of medicinal chemistry. Synthesis of new derivatives of carboranes, polyhedral borate anions and metallacarboranes and their evaluation as potential antitumor agents are presented here based on own results and literature data. Particularly results on synthesis and *in vitro* antitumor activity of organotin derivatives of carboranes and synthesis of new polyhedral boron compounds as potential BNCT agents are described.

Key words: carborane, dodecaborate anion, cobaltacarborane, antitumor agents, BNCT.

Introduction

The main direction of application of polyhedral boron compounds currently is medicine. Synthesis of new derivatives of polyhedral boron compounds as potential antitumor agents is the subject of this lecture. There are four main potential applications of polyhedral boron compounds in medicine:

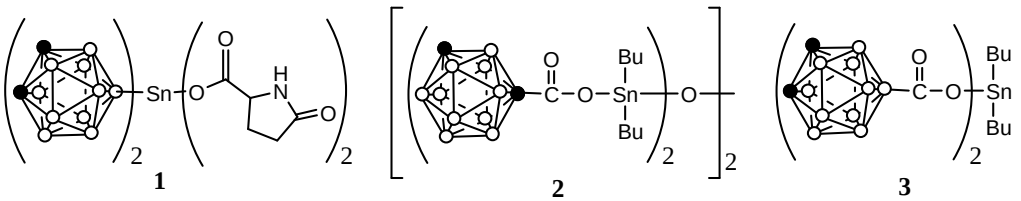
a) Boron Neutron Capture Therapy (BNCT) of cancer [1,2]
b) Carboranes as pharmacophore [3]
c) Radionuclide diagnostics and therapy of cancer, where these compounds can be used for attachment of radionuclide labels to various cancer-targeting biomolecules [4,5]
d) Antitumor activity of metal-containing carboranes [6].

These four main directions of application of polyhedral boron compounds in medicine have been recently discussed in our reviews [5,6]. Here our recent results and some literature data on synthesis and antitumor activity of metal-containing carboranes, as well as the preparation of new derivatives of carboranes, dodecaborate and cobaltacatborane for BNCT will be presented.

Antitumor activity of metal-containing carboranes

When the antitumor activity of cisplatin, *cis*-$Cl_2Pt(NH_3)_2$, was discovered, several research groups started to investigate the possible therapeutic applications of other metal-based compounds. Derivatives of cisplatin containing carborane fragment were prepared and their activities were observed. The *in vitro* cytotoxicity of {*cis*-$[NH_3Cl_2PtNH_2](CH_2)_3\}_2$-1,7-$C_2B_{10}H_{10}$ and $\{[Cl(NH_3)_2PtNH_2(CH_2)_3]_2$-1,7-$C_2B_{10}H_{10}\}^{2-}$ $2(TfO^-)$ was screened against a range of tumor cell lines in order to determine whether the complexes exhibited a cytotoxic effect in absence of neutrons. The cell lines included L1210 murine leukaemia cells and its cisplatin-resistant variant (L1210/DDP), along with the cisplatin-sensitive (2008) and –resistant (C13*5) human ovarian carcinoma. The mechanism of inducing cell death is unknowm at the present time, but it is reasonable to assume that the effects are the result of avid DNA-binding as the complexes are capable of binding to plasmid DNA. Charged compound was consistently more active than neutral one in all cell lines that were examined. This may be not only be due to enhance the aqueous solubility of the complex but perhaps also enhance its DNA-binding affinity via electrostatic interactions. In both cisplatin-sensitive cell lines L1210 and 2008, platinum-carborane complexes were not as effective as cisplatin at inhibiting cell growth [7].

Some organotin derivatives of carboranes were synthesized and their antitumor activities were measured. A carborane moiety $C_2B_{10}H_{12}$ has about the same size as a phenyl ring but is spherical instead of planar. The replacement of an aromatic phenyl ring by a hyper-aromatic carboranyl moiety is routinely used in the field of boron-based metallotherapeutic agents. In some compounds, the tin atom was linked directly to one of the boron atom of the carboranyl moiety [8]. In other cases di(n-butyl)tin carboranecarboxylates were synthesized [9-11] (Scheme 1).

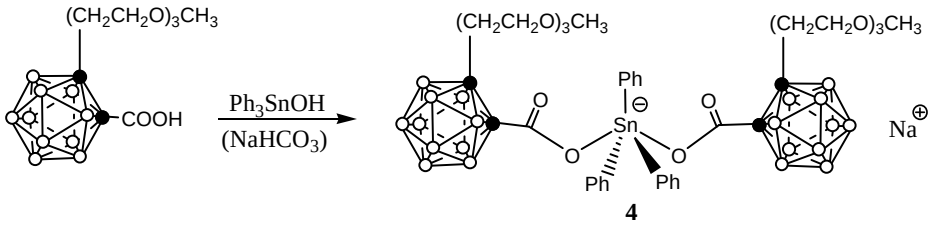


1 **2** **3**

Scheme 1

The dimeric bis[(1,7-dicarba-*closo*-dodecaborane-1-carboxylato)-di-*n*-butyltin] oxide, was obtained from a 1:1 condensation of dibutultin(IV) oxide with 1,7-carborane-1-carboxylic acid (Scheme 1).
Bis(1,2-dicarba-*closo*-dodecaborane-9-carboxylato)di-*n*-butyltin, $(1,2\text{-}C_2B_{10}H_{11}\text{-}9\text{-}COO)_2SnBu_2$, the first carborane-based organotin compound where the carborane cage is linked to the carboxylic moiety *via* a boron atom, was obtained by the 2:1 condensation of 1,2-carborane-9-carboxylic acid with di-*n*-butyltin(IV) oxide (Scheme 1) [11].

In order to increase the water solubility of a carborane-based organotin compound an attempt to synthesize such compound with polyoxaalkyl chain linked to the carboranyl moiety was accomplished (Scheme 2) [6,12].



Scheme 2

The novel sodium bis[2-(3',6',9'-trioxadecyl)-1,2-dicarba-*closo*-dodecaborane-1-carboxylato]triphenylstannate was synthesized by the condensation of triphenyltin(IV) hydroxide with 2-(3',6',9'-trioxadecyl)-1,2-dicarba-*closo*-dodecaborane-1-carboxylic acid followed by crystallization in the presence of sodium bicarbonate that permits the isolation of crystals from the mixture. The antitumor activities ($ng{\cdot}ml^{-1}$) of all carborane-based organotin compounds prepared were determined *in vitro* against six well characterized human tumor cell lines: MCF-7 and EVSA-T (two breast cancers), WiDr (a colon carcinoma), IGROV (an ovarian cancer), M19 MEL (a melanoma), A498 (a renal cancer) together with those of some reference compounds used clinically (Table 1) [6,12]. Tin-containing carboranes are significantly more active than the clinically used 5-fluorouracil, *cis*-platin and carboplatin, and sodium bis[2-(3',6',9'-trioxadecyl)-1,2-dicarba-*closo*-dodecaborane-1-carboxylato]triphenyl-stannate is comparable with methotrexate and doxorubicin. It exhibited a highest cytotoxicity, perhaps because its water solubility was increased by the presence of the polyoxa substituent, and probably due to the fact that the compound was a salt.

Boron neutron capture therapy

The leading in application of polyhedral boron hydrides in medicine belongs to Boron Neutron Capture Therapy (BNCT)– a binary cancer treatment

based upon the interaction of two relatively harmless species, a ^{10}B nucleus and a thermal neutron. The concept of BNCT was described widely [1-3].

Table 1. *In vitro* antitumor activity (ng ml^{-1}) of some tin-containing carboranes in comparison with clinically used agents.

Compounds	Cell lines					
	MCF-7	EVSA-T	WiDr	IGROV	M19	A498
1	60	48	410	3	30	110
2	45	38	290	110	110	140
3	146	142	429	139	174	195
4	44	38	37	39	39	45
cis-platin	1400	920	1550	230	780	1200
5-fluorouracil	350	720	440	850	310	340
methotrexate	15	26	7	20	18	16
doxorubicin	25	13	18	150	21	55

The selective concentration of the ^{10}B nuclei within tumor cells, followed by their capture of thermal neutrons, should result in localized destruction of the malignant cells in the presence of the neighboring normal cells. The main requirements for BNCT agents are a selective accumulation of ^{10}B in tumor cells, achieving concentration of agent in tumor in the range of 20-35 μg $^{10}B/g$, a sufficiently low toxicity and solubility in water. The first requirement is a selective accumulation of agent in cancer cells. The absence of an adverse effect on the surrounding healthy tissues is attributed to the fact that the thermal neutron capture cross-sections of elements involved in the human tissues are 4—7 orders of magnitude smaller than that of the ^{10}B isotope.

The so called “first generation” boron carriers (sodium borates, boric acid, and its derivatives) do not satisfy the above-mentioned criteria both with respect to selective accumulation in the tumor and the achievement of the desired therapeutic concentration. Since the most known polyhedral boron compounds contain 10-12 boron atoms, they attracted attention of scientists as BNCT agents shortly after discovery. Their use makes it easier to achieve the required therapeutic concentration of boron atoms in cell. They also meet other requirements: they are non-toxic, stable and some of them are water-soluble.

The only two BNCT agents currently used for clinical trials are L-*p*-dihydroxy-borylphenylalanine (BPA), a boronated amino-acid analog, and disodium mercaptoundecahydro-*closo*-dodecaborate $Na_2B_{12}H_{11}SH$ (BSH). They both are so-called “second generation” of BNCT agents [13-15]. Clinical treatments with BNCT started in Japan in 1968 mostly by H.Hatanaka. Using both BSH and BPA in 20 years he treated more than 200 patients, and many of

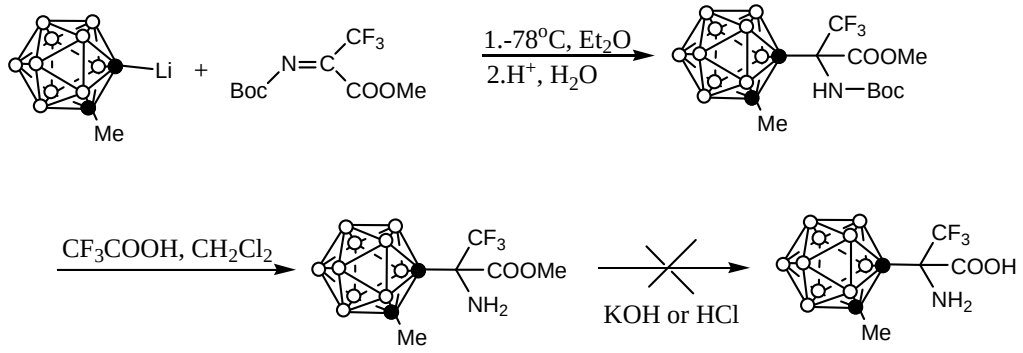
them were long-term survivors of malignant brain tumors although most of them were so seriously ill that could not be treated by other methods [16].
In 1990-s clinical trials has started in two centers in the USA [17,18] and later on in some European countries [19].

However both these drugs BPA and BSH are far from ideal. Therefore many different types of boron containing compounds have been designed and synthesized for testing as BNCT drugs over the past 30 years. In order to fulfill the requirements for the BNCT agents these new compounds ("third generation" of BNCT agents) should consist of a boron-containing part connected via a hydrolytically stable linkage to a tumor-targeting part, responsible for delivering of boron fragment to the tumor cell (boron carrier) and its retention there [2]. A spacer between boron moiety and boron carrier needs to prevent steric and electronic influence of boron part (especially in the case of boron polyhedron) on the capability of tumor targeting entity to be accumulated selectively in tumor. A great number of papers were published recently concerning synthesis of potential BNCT agents where polyhedral boron fragment is connected with some biomolecules, responsible for delivering boron fragment to the tumor cell. A lot of boron conjugates with such boron carriers as amino acids and peptides, carbohydrates, lipoproteins and liposomes, porphyrins and phthalocyanines, the precursors of nucleic acids such as pyrimidines, purines, nucleosides and nucleotides were synthesized during the period 2000-2007 and evaluated as agents for BNCT. There were more than 1000 publications on this subject. A complete review on this subject was published recently [5]. So, in this paper we present mostly our results on synthesis of some functional derivatives of polyhedral boron compounds and their conjugates with boron carriers as potential agents for BNCT.

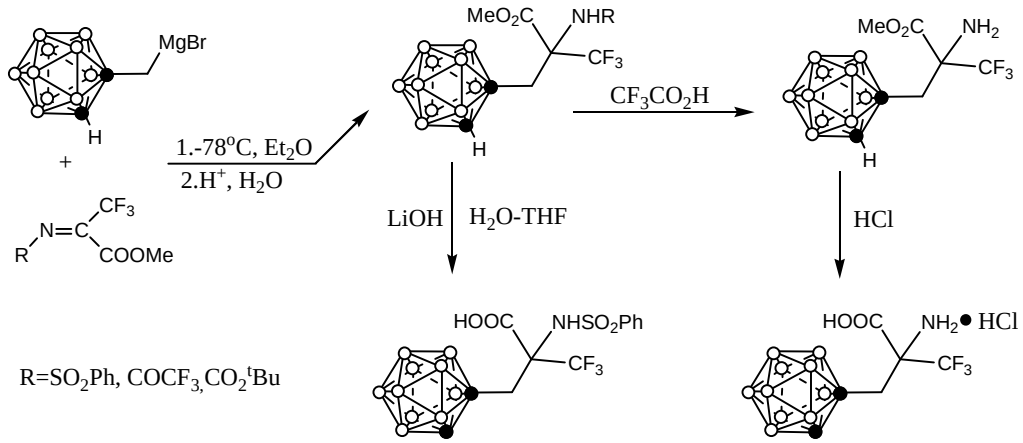
Since L-*p*-dihydroxy-borylphenylalanine (BPA) is one of the successful BNCT agents, a great deal of work has been done on synthesizing amino acids linked with a polyhedral borane moiety and their evaluation as potential agents for BNCT.

New alanine derivatives containing both the *o*-carboranyl and trifluoromethyl groups were synthesized by the reaction of (methyl-*o*-carboranyl)lithium or *o*-carboranylmethylmagnesium bromide with N-protected methyltrifluoropyruvate imines to give correspondingly N-protected α-carboranyl- or β-carboranyl- α-trifluoromethyl-α-amino acid esters (Schemes 3, 4) [20,21]. In both cases the protected groups were removed by the reaction with trifluoroacetic acid in dichloromethane to give α-carboranyl- or β-carboranyl- α-trifluoromethyl-α-amino acid esters (Schemes 3, 4). Methyl ester of β-carboranyl-α-trifluoromethyl-α-amino acid was refluxed in 6M HCl resulting in corresponding amino acid as hydrochloride (Scheme 4). Hydrolysis of the ester group without deprotection of nitrogen was performed for β-

carboranyl-α-amino acid ester by the reaction with lithium hydroxide giving rise to *N*-protected acid (Scheme 4) [21].



Scheme 3



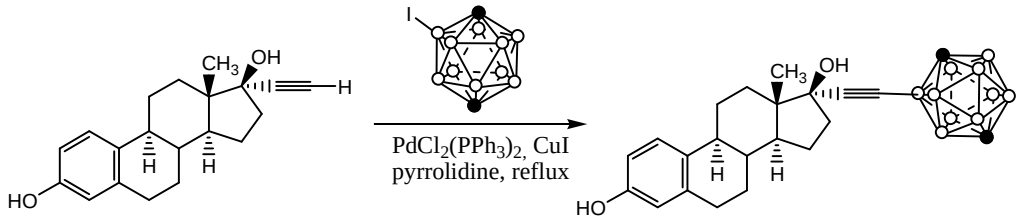
Scheme 4

Such amino acid containing free carboxyl group and protected amino group could be used as the precursor for the synthesis of peptides required for BNCT. In the case of using 1R-(-)-menthyl group as protecting group at the nitrogen atom a mixture of diastereomers has been obtained. A structure of one of diastereomers was characterized by X-ray method [21].

Several biomolecules contain heterocycles in their molecules. In order to conjugate carboranyl group with heterocycles the Pd-catalyzed cross-coupling reactions of 9-iodo-*m*- and 2-iodo-*p*-carboranes with heterocyclic organozinc compounds were studied. At the first time the furyl, thienyl, indolyl, pyridyl, quinolyl B-derivatives of the carboranes were isolated [22].

Based on methods of metallocomplex catalysis, carboranylalkynyl derivatives of estradiol have been synthesized, in other words, steroid fragment was introduced to carborane molecule (Scheme 5) [23]. Water-soluble functionalized derivatives of the dodecahydro-*closo*-dodecaborate and cobaltbis(dicarbollide) anions are promising candidates for BNCT. It was

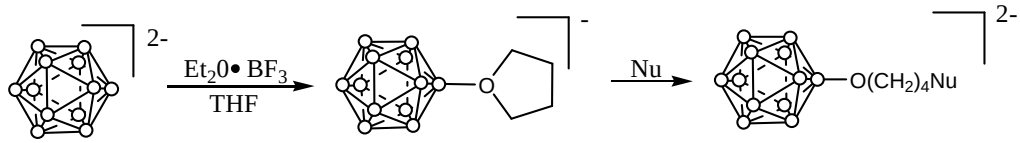
found that synthesis of their oxonium derivatives is one of the most powerful ways to introduce the reaction centre into boron cage.



Scheme 5

Oxonium derivatives of dodecaborate were formed under the reaction of dodecaborate with THF or dioxane in the presence of Lewis acid such as BF_3 etherate. Recently a review on preparation and transformation of such oxonium derivatives was published [24].

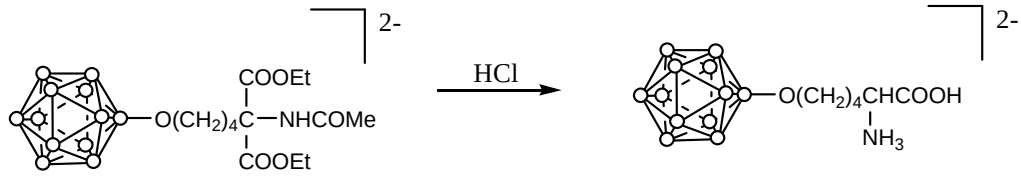
Reactions of dodecaborate oxonium derivatives with various O- and C-nucleophiles gave rise to a great variety of boron cluster derivatives with different functional groups (Scheme 6).



Nu=OH, CN, $CHS_2(CH_2)_3$, $N(CO)_2C_6H_4$, $CNHCOMe(COOEt)_2$

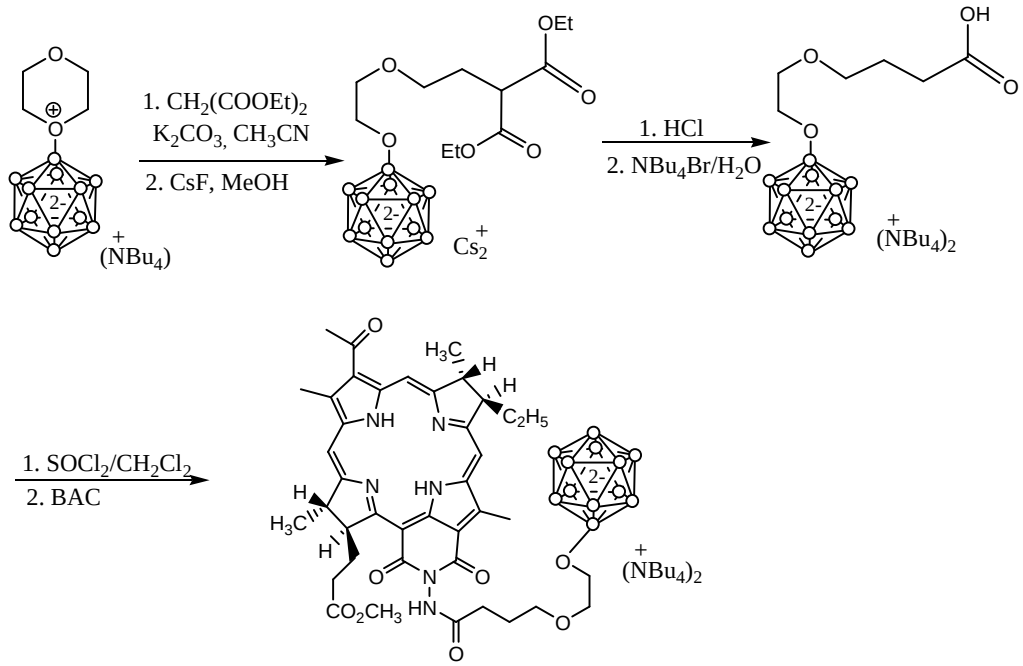
Scheme 6

Boron-containing amino acids (Scheme 7) [25] and carbohydrates [26-29] were prepared using this method.



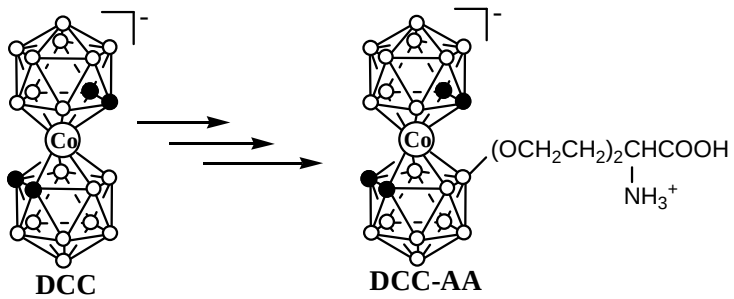
Scheme 7

Starting from the dioxane-based oxonium derivative of dodecaborate anion, conjugates of dodecaborate anion with phthalocyanine [30] and N-iminocycloimide bacteriochlorin *p* (BAC) (Scheme 8) [31] were synthesized.



Scheme 8

Similar to $[B_{12}H_{12}]^{2-}$ the dioxane-based oxonium derivative of cobalt bis(dicarbollide) $[3,3'\text{-}Co(1,2\text{-}C_2B_9H_{11})_2]^-$ (DCC) gives amino acid (DCC-AA) attached to cobaltacarborane cage through bisethyleneoxide spacer (Scheme 9) [32]. This amino acid was proposed as a new boron moiety for BNCT agents. This moiety contains more boron atoms per molecule than carboranes or $[B_{12}H_{12}]^{2-}$. In BNCT experiment *in vitro* against melanoma B-16 the amino acid prepared from cobalt bis(dicarbollide) was shown to increase killing effect of neutron radiation on tumor cells. Cobalt bis(dicarbollide) and its derivatives are water-soluble as sodium salts, and at the same time, are sufficiently hydrophobic to translocate in some way across the phospholipid bilayer membranes [33,34].



DCC **DCC-AA**

Scheme 9

Acknowledgements
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FLUORESCENTLY LABELED METALLACARBORANES: SOLUTION BEHAVIOUR AND INTERACTION WITH SERUM PROTEINS

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Summary

Series of cobalt(III) bis(dicarbollide) conjugates with different fluorophores was prepared. Cell internalization and solution properties of novel molecules were tested and the process of molecular association was monitored using light scattering, atomic force microscopy a fluorescence spectroscopy. The possibility of aggregate decomposition by action of serum albumin was demonstrated.

Key words: metallacarborane, cobalt(III) bis(dicarbollide), fluorescence, serum albumine, aggregation.

Introduction

Metallacarboranes of cobalt(III) bis(dicarbollide) type absorb stongly UV light, however their inherent fluorescence is not known. In an attempt to find possibilities for fluorescence monitoring of cell entry and action of HIV protease inhibitors containing this moiety [1], we focused on development and characterization of metallacarbones with attached fluorescent probes.

Variety of such fluorescent metallacarborane conjugates was prepared and characterized, including porphyrine, fluorescein, dansyl and Alexa probes (for structures and synthetic schemes see Fig. 1).

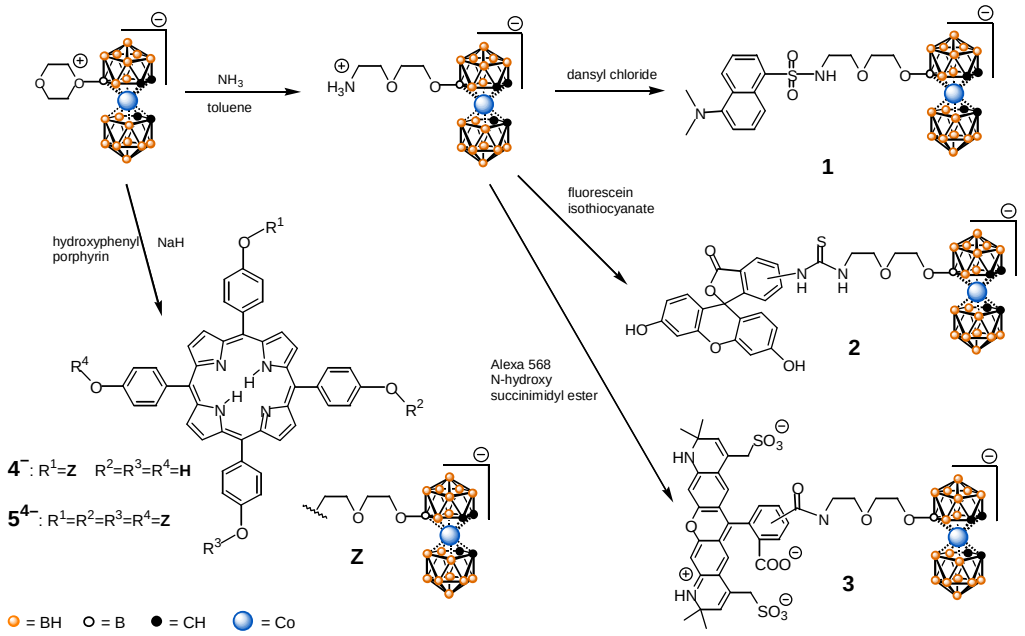

Figure 1. Selected metallacarborane conjugates containing fluorescent probes and the synthetic schemes used for their preparation.

Biological activity and cell internalization

All the compounds inhibited HIV protease, however their inhibition efficacy differed in two orders of magnitude. For study of cellular entry, compounds **2** and **3** were selected. They represent most hydrophilic compounds from studied series and their fluorescence intensity is constant in time, in contrast to hydrophobic **1**, **4** and **5** that strongly aggregate in aqueous solutions.

Cell internalization of compounds **2** and **3** is depicted on Fig. 2. The experiment was performed on two different media (PBS and IMDM). Already one hour after addition the compounds were effectively internalized from both media. As obvious from the pictures, **2** and **3** are not accumulated in cell nuclei.

Porphyrin conjugates of metallacarboranes

Thorough physico-chemical study of metallacarborane-porphyrin conjugates behaviour in solutions was performed. The dependences of absorption and fluorescence spectra on pH and solvent type, the triplet state spectra and yields of triplet oxygen were measured for conjugates **4** and **5**. The aggregates present in solutions were observed using QELS and AFM [2]. Similar structures and their tendency to aggregate were reported also from Vicente and coworkers [3-6].

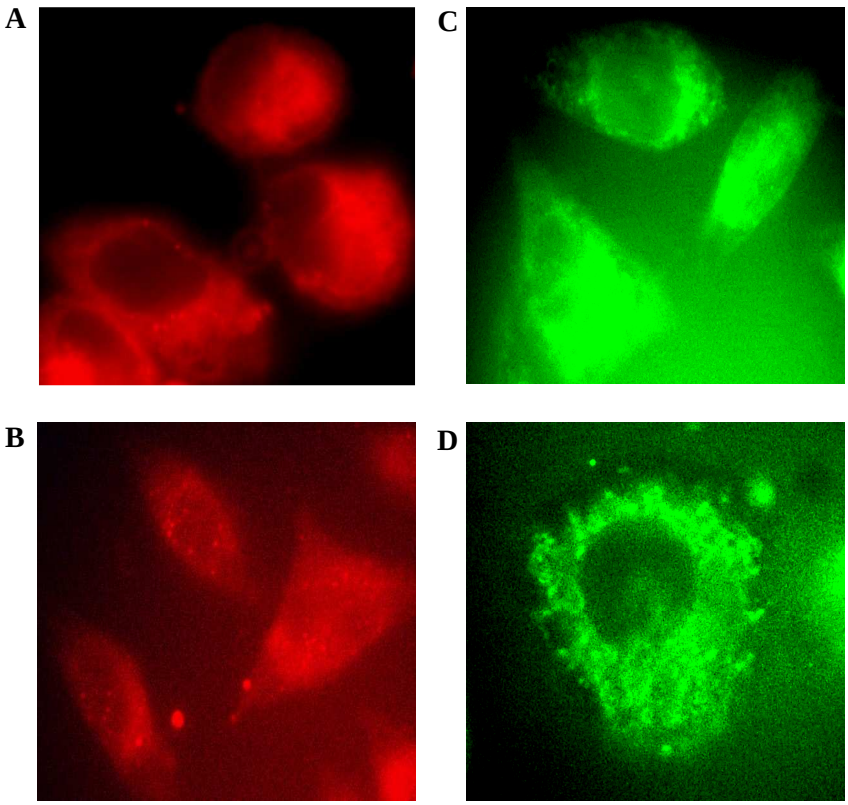

Figure 2. Internalization of compounds **2** and **3** into HeLa cells visualized using fluorescence microscopy. **(A)** Compound **3** in PBS medium and **(B)** in IMDM medium. Detection: excitation filter 510 – 550 nm, emission filter 590 nm. **(C)** Compound **2** in PBS medium and **(D)** in IMDM medium. Detection: excitation filter 460 – 490 nm, emission filter 510 nm.

Absoption and fluorescence spectra in methanol are characteristic for this type of porphyrins (Soret band at 418 nm and four Q-bands at higher wavelenghts). The absorption band at 311-313 nm is caused by presence of matallacarborane moiety in molecule (see Fig. 3 A-a a B-a). After acidification of solution by formic acid, the protonization of molecule occurs and characteristic alteration of spectra are observed (red shifted Soret band and reduction of Q-band number; Fig. 3 A-b a B-b).

In contrast, the behaviour in aqueous solution is very different: due to aggregations effects, strong hypochromic effect and widening on Soret band was observed together with Q-bands disappearing. Also the fluorescence ofwater solution vanished. The acidification causes further intensive aggregation that could be reversed by alkalization of the solution.

The presence of aggregates in solutions was confirmed using QELS and AFM. Stable particles of diameters about 250 – 300 nm were observed. However, after acidification, the interaction of metallacarborane anions with positively charged protonized porphyrins lead to precipitation of solutions.

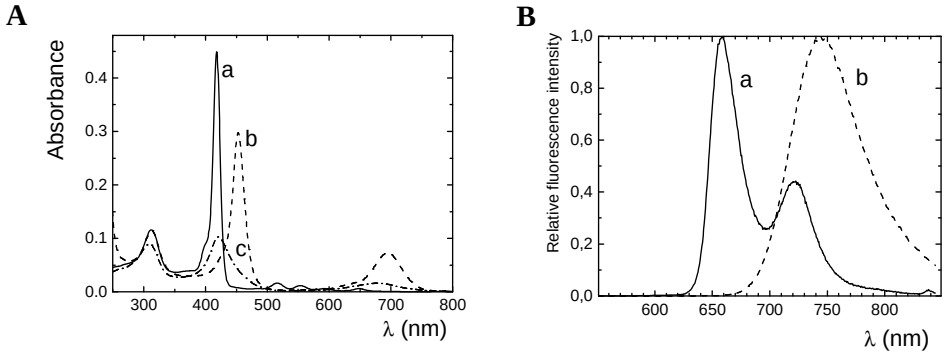

Figure 3. **(A)** Absorption spectra of **4** (a) in methanol (b) in methanol ofter acidification by HCOOH (0.3 % v/v), (c) in phosphate buffer (20 mmol/L) pH = 7.1. **(B)** Corrected normalized fluorescence emmision spectra of **4** (1.1 μmol/L) (a) in methanol, excitation 418 nm, (b) in methanol ofter acidification by HCOOH (0.3 % v/v), excitation 453 nm.

We found also that the strong aggregators containing metallacarborane moiety could be deaggregated by action of serum albumins. As example, titration curve of compound **1** by bovine serum albumin is shown on Fig. 4. The increase of fluorescence indicates the deaggregation of **1**-aggregates caused by consecutive complexation to serum albumine. The deaggregation process was confirmed also using QELS.

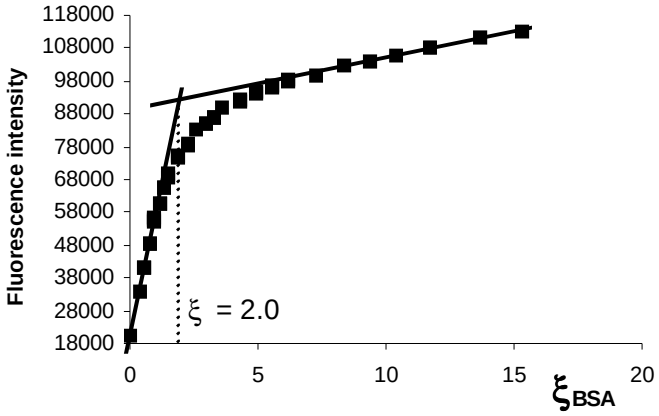

Figure 4. Dependence of fluorescence intenzity of dansyl fluorophore conjugated with metallacarborane (compound **1)** on molar excess of bovine serum albumine ξ_{BSA}. Experiment was performed in 10 mM Hepes buffer pH 7.0, excitation 328 nm, emmision 474 nm.

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DNA PRECURSOR ENZYMES AND BORANO NUCLEOSIDES

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Summary

Boronated nucleosides could be ideal for targeting tumor cells in boron neutron capture therapy (BNCT) provided that they could be metabolized and accumulate selectively in such cells at sufficient levels. This presentation summarizes the current knowledge concerning borano nucleosides and DNA precursor enzymes with emphasis on their structure activity and selective expression in tumor cells compared to normal tissues. The high capacity of thymidine kinase 1 (TK1) to phosphorylate N3 substituted carboranyl nucleoside analogs with varying substitutions will be reviewed as well as the recent great advances in understanding of TK1 structure and function relationships and cell cycle regulation. The successful use of one of the N3 analogs in BNCT of a brain tumor model in rats will be described as well as the potential use of other DNA precursor enzymes as targets. The results presented here may inspire the design of new nucleoside analogs in which the bulky carborane cage is projected out side of the active site of key metabolizing enzymes through a tether.

Key words: Carboranyl deoxynucleosides, structure function studies, deoxynucleoside kinase, selective trapping.

Introduction

The stability and physiochemical versatility of carboranes led to their frequent use in Boron neutron capture therapy (BNCT) and in other areas of drug design [1]. BNCT is a therapeutic method based on the selective delivery of non-radioactive boron-10, followed by irradiation with low energy thermal neutrons to the site of a malignant tumor. The resulting (^{10}B[n, α]^{7}Li) nuclear capture and fission reactions yield α particles and ^{7}Li nuclei with high linear energy transfer, the approximate length of a cell. Administration of the ^{10}B agent is adjusted to give a maximal difference between boron concentrations in the tumor, normal tissues and blood. For BNCT to be successful, there must be an accumulation of ^{10}B in the tumor that reaches ~20 µg/g tissue, combined with low levels in normal tissues. Several delivery agents have been tested in

order to get selective accumulation of ^{10}B within tumor cells and boronphenylalanine (BPA) and sodium borocaptate (BSH) have been used in the clinic [2]. BNCT has given positive clinically results in case of high grade gliomas, malignant meningiomas, melanoma, and carcinomas of the head and neck [3-5].

DNA precursor enzymes

A second approach for selective delivery, which is the subject of this presentation, is based on targeting DNA precursor enzymes (Fig 1). The primary target is cytosolic thymidine kinase 1 (TK1), which have been demonstrated to accept several types of borano nucleoside analogs [6-9]. This enzyme is selectively expressed at high levels in proliferating cells and thus in several types of malignancies [10]. The salvage of external nucleosides and nucleobases are initiated by transport *via* several types of membrane carriers (equillibrative and concentrative transporters, respectively, Fig 1) [11] into the cytosol. The phosphorylation of the nucleosides by deoxynucleoside kinases give negatively charged products that can not be transported out of the cells and this processes have been called kinase-mediated trapping (KMT) [12].

Uptake of nucleo-bases is also part of the salvage pathway but in this case the formation of ribonucleotides occur through the activities of phophoribosyltrans-ferases using 5-phophoribosyl-1-pyrophosphate (PRPP) as co-substrates (Fig 1). The products of these reactions can serve as DNA precursors via ribonucleotide reduction. However, these pathways will not be further discussed here. There are four deoxynucleoside specific kinases (dNK;s) in animal cells i.e. cytosolic TK1 and dCK and mitochondrial thymidine kinase (TK2) and deoxyguanosine kinase (dGK). The latter two kinases are essential for the production of mitochondrial DNA precursor particularly in the resting cells in tissues such as liver, muscles and nerves [10,13]. These enzymes are also responsible for the activation of nucleoside analogs used anticancer- and antiviral nucleoside analogues and they are involved in mitochondrial side effects associated with anti HIV chemotherapy [10]. The next step in deoxynucleotide biosynthesis is carried out by nucleoside monophospahete kinases (Fig 1), which usually are specific both for the base and the sugar moieties of the substrates i.e. deoxy- or ribonucleoside kinases. However, there are several exceptions to this rule with enzymes demonstrating a broader specificity [14].

The main source of DNA precursor is derived from ribonucleoside diphosphates via the allosterically controlled enzyme ribonucleotide reductase (RR) (Fig 1) [15]. However, thymidine deoxynucleotides have a separate synthetic pathway based on methylation of dUMP by thymidylate synthase [16]. Both of these enzymes are cell cycle regulated and up-regulated in proliferating cells and potentially interesting target enzymes in BNCT.

Nucleoside monophosphate kinases catalyse an essential step both for the *de novo* and in the salvage synthesis of DNA precursors in all organisms.

The final step in this pathway is catalyzed by nucleoside diphosphate kinases (NDPK) (Fig. 1), which are accepting deoxy- and ribonucleoside diphosphate and both purine and pyrimidines. There are several iso enzymes of NDPK with different sub-cellular location and tissue specificities [7].

Through the combined actions of these transporters and kinases mono-, di-, and triphosphates of nucleoside analogues can selectively accumulate in millimolar concentrations in malignant or virally infected cells [18]. The triphosphates are usually the active metabolites responsible for antiviral or cytostatic activity by inhibiting polymerases or reverse transcriptases leading to termination DNA chain polymerization. However, consequently these enzymes are also responsible for most of the toxic side effects associated with nucleoside analogue drugs, e.g. bone marrow or intestinal epithelium damage [19]. TK1 is an ideal target in KMT since it is almost exclusively expressed during the S-phase of the cell cycle [12]. In malignant cells TK1 activity is also high in the G2 phase of the cell cycle and serum TK1 is an important prognostic indicator for tumor proliferation and invasiveness as well as an early warning sign for tumor recurrence [20,21].

Recently, an M phase dependent ubiquitination process was shown to be responsible for rapid degradation of TK1 leading to the very low levels of enzyme found in G0 or early G1 cells [22]. In addition, also transcriptional and translational regulation of TK1 occurs as well as protein kinase dependent phosphorylation [10,23]. Thus, TK1 expression is regulated at multiple levels and these processes are both tissue and species dependent. The fact that TK1 leaks out from rapidly proliferating cells such as cancer cells, has triggered the development of several assay of serum TK1 that have found use in clinical cancer management [20,21,24]. The recent substantial increase in our knowledge regarding the structure and function of TK1 will be described below in relation to its activity towards borano nucleosides. A second target in KMT is dimeric dCK, which belongs to another enzyme family than TK1. This enzyme has a broad substrate specificity accepting both pyrimidine and purines including many important antiviral and cytostatic nucleoside analogues. There is about 30-40% sequence homology between dCK, dGK, and TK2 and their structures confirm these similarities [10,13]. The overall structure of TK2 remains to be determined but is most likely similar to dGK.

The substrate specificity of dCK has been difficult to study because it shows two apparent K_m and V_{max} values in the nucleoside saturation curves, and the Hill plots show a negative coefficient. The reaction conditions (e.g. the nature of the phosphate donors, UTP instead of ATP) as well as variations in the storage and treatment of the enzyme affect the kinetics observed [10]. Several new dCK crystal structures were described very recently [25]. The

ternary enzyme complexes were complexed with two enantiomeric forms of dAdo (D-dAdo, or L-dAdo), with either UDP or ADP bound to the phosphate donor site. The structures containing UDP showed open state of dCK in which the nucleoside, either D-dAdo or L-dAdo, was bound in a non-productive manner. In contrast, the complexes with ADP, with either D-dAdo or L-dAdo, adopted a closed and catalytically competent conformation. The different states adopted by dCK in response to the nature of the nucleotide phosphate donor were also detected by tryptophan fluorescence experiments. Furthermore, these results supported the conclusions obtained in previous biophysical studies demonstrating a change in dCK conformation due to substrate binding [25,26].

Recent studies using spontaneous and directed evolution combined with detailed kinetic analysis have provided a better understanding of the substrate selectivity of dCK [27]. If Arg104 and Asp133 in the active site of dCK were changed to Gln104 and Gly133 the mutant dCK had a very broad specificity including Thd as substrate and it also had elevated turn-over rates. These results demonstrate the key role that these amino acids residues play in directing the substrate specificity and explain why this family of dNK;s has structural similarity but greatly varying specificities. The expression of dCK in different cells and tissues as well as during the cell cycle has shown that dCK is up-regulated in rapidly proliferating cell such as cancer cells but the extent of up-regulation is significantly lower than TK1 [10]. There is also a tissue specific pattern of expression. Lymphatic cells as well as resting lymphocytes have higher dCK levels than other cell types. An apparent “stress” related stimulation of dCK activity is observed in cells treated with cytostatic and gentotoxic agents and this was recently shown to be due to phosphorylation of Ser74 [28,29] by an as yet unidentified protein kinase.

Borano nucleosides and DNA precursor enzymes

Previous studies on the design and synthesis of boron containing analogues of nucleosides as boron delivery agents for NCT were initiated by Soloway and co-workers [30,31]. The rationale was based on the fact that cancer cells have increased requirements for nucleic acid precursors, due to higher proliferation compared with normal cells, and an increased involvement of the salvage pathways of DNA precursors synthesis. A panel of 3-carboranyl thymidine analogues (3CTAs) was synthesized and evaluated as substrates of TK1, as will described further below and as recently reviewed in detail [9].

However, the first 5-substituted carboranyl nucleoside, 5-(1-ocarboranyl)- 2´-deoxyuridine (CDU), was reported by Yamamoto et al. [32]. They introduced the carborane cluster at the 5-position of dUrd because this substitution e.g. iodine and bromine have resulted in known nucleoside pro-drugs, (e.g. 5-iodo- and 5-bromo-2´-deoxyuridine) which have anti tumor activity.

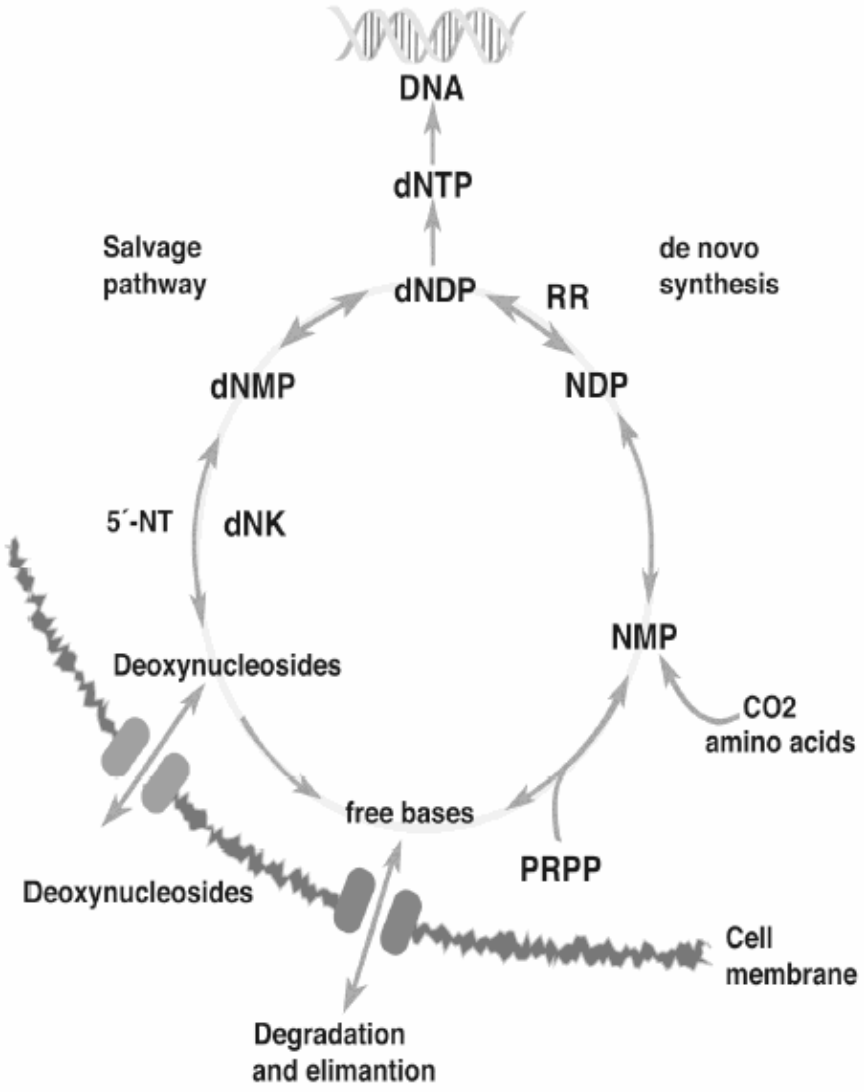

Figure 1. Schematic representation of DNA precursor metabolism in mammalian cells. The abbreviations and the enzyme steps are described in the text.

CDU was evaluated as a BNCT candidate by Schinazi and his coworkers [33,34] and shown to be phosphorylated by TK2 but not by TK1 [35]. CDU monophosphate was detected in human CEM and PBM cells [33] with low apparent toxicity. There was a complete metabolism of CDU into triphospahtes and incorporation into DNA but the degree of Thd substitution of CDU was relatively low (1.8 %) [36]. Positive results were reported for experimental rat brain tumors treated by neutron capture therapy after application of CDU [37].

In case of 3CTAs enzyme and cell culture studies with several different libraries of these analogs have been performed since 1999 and the results reviewed recently [9]. The analogs are designated N4, N5, and N7 and the corresponding 3-dihydroxypropyl derivatives, N4-2OH, N5-2OH and N7-2OH [37]. Of these compounds, N5-2OH (3-[5-{2-(2,3-dihydroxyprop-1-yl)-*o*-carboran-1-yl}pentan-1-yl]thymidine) (Fig. 2) had the most favorable overall properties, which included high phosphorylation by TK1, low toxicity, and high cellular uptake and retention [8,9,38]. Based on these properties, *in vivo* studies were initiated with N5-2OH as a boron delivery agent for BNCT in tumor bearing mice and rats. Validation of TK1 as an appropriate molecular target was demonstrated using the TK1 positive L929 tumor model. Since brain tumors have been the major focus of our experimental and clinical studies of BNCT we have investigated N5-2OH as a boron delivery agent using the F98 and RG2 rat glioma models. N5-2OH and other 3CTAs were taken up and retained in the different TK1-containing wild-type cell lines (F98, RG2, L292 and CEM) but only to a significantly lesser degree in the TK1 deficient counterparts (L929, CEM). In contrast, BPA accumulated in L929 wild-type and L929 TK1 negative cells to the same extent. Boron concentration levels in TK1-expressing wild type cell lines after exposure to N5-2OH exceeded significantly those necessary for BNCT. The *in vitro* toxicity of 3CTAs was generally moderate to low [8,9,39].

BNCT experiments were recently conducted by Barth et al [39] with tumor -bearing rats and mice. These received N5-2OH, either administered with convection-enhanced delivery of macromolecules into the brain tumors or intratumoral injection into subcutaneous tumors [40]. The boron concentrations in subcutaneous L929 TK1 positive tumors was 2.7 fold higher than in the corresponding TK1 negative tumors. Following BNCT, mice bearing TK1 wild type tumors had a 15-fold inhibition in tumor growth compared to TK1 negative matched controls. Tumor boron concentrations for F98 and RG2 gliomas were 17.3 and 27.6 µg/g, respectively. Normal brain and blood had undetectable levels of boron. After BNCT with N5-2OH the mean survival times of F98 and RG2 glioma bearing rats were 38 d. and 46 d., respectively. The mean survival times of irradiated and untreated controls were 31 d. and 25 d. for F98 glioma bearing rats and 28 d. and 24 d. for the RG2 glioma. Thus, N5-2OH apperars to be effective in treating rat gliomas in a TK1-dependent manner and these results clearly support the KMT concept as basis for BNCT.

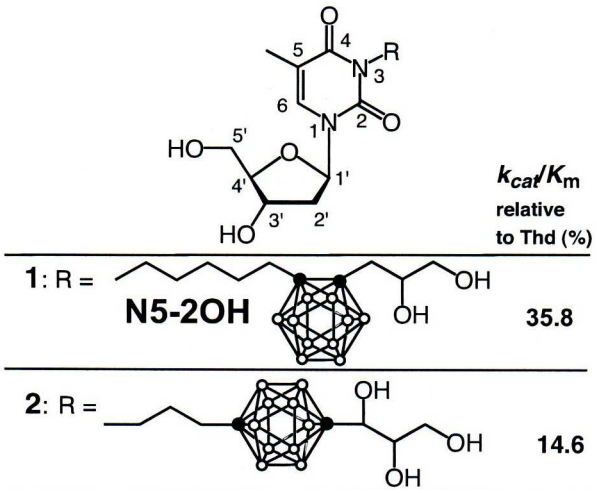

Figure 2. The structure and activity of two 3CTA:s with human TK1.

The substrate specificity of DNA precursor enzymes towards borano nucleosides.

Recent structure activity studies with TK1 have revealed several important new facts about TK1. The crystal structures of human hTK1 in complex with dTTP have been determined as well as several bacterial TK:s in complex with Thd, dTTP, TMP/ADP [41,42] and generally the show highly similar folds and limited variations in the active site structures. However, two basic states of TK1 were found: One type is a "closed" TK forms which is found in case of the feed-back inhibitor complexes and TMP/ADP reactant complexes. The second form is an "open" or "semi-open" apo TK1 form [42]. As with many other nucleoside- and nucleotide kinases, binding of substrates and the phosphate donor (ATP) in the TK1 enzyme family is associated with a large conformational change from the open unoccupied form, over a closed form involving Thd and ATP with its beta and gamma phosphate in the P-loop ready to be transferred to the 5´-OH of Thd (Fig 3). The so called "lasso loop" interacting with and moving down over the Thd base become fully ordered only in the in this type of catalytic competent complex. N3 of Thyd is oriented towards the entrance gap between the lasso loop and the strands of the base domain of the active site. Thus, the carboranyl cage of 3CTAs, attached via a methylene tether to N3, will be projected out of the active site lasso loop while the 5′-OH group at the other end of the molecules can reach the catalytic residues involved in phosphoryl transfer (Fig 3).

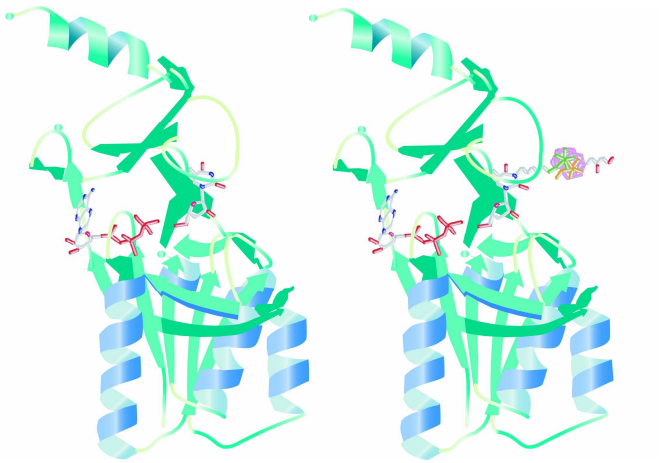

Figure 3. Schematic drawings of TK1 in complex with Thd and ATP (right) and N5-2OH and ATP (left) based on the modeling results in reference [43] and the structures presented in reference [42].

The fact that the borano cage is situated out side of the active site of TK1 explains the structure activity studies which showed that there was an optimum length of the connecting tether, but also a very large acceptance of variations in the type of carborane cage and substitutions patterns of the cage. The optimum length was in case of TK1 five methylene groups [38]. The principal of placing the carboranyl cage or other bulky substitutions out side the active site ("the out of site principle") could be generally applicable in order to avoid unwanted spatial constrains or interference with key interactions in the active site.

Another major factor involved in determining the activity of the carboranyl nucleoside analogues was lipophilicity and water solubility. 3CTAs containing a dihydroxypropyl group at the second carbon of the carboranyl cage were better substrates than the corresponding analogs without this substitution [38]. The stability of the N5-2OH monophosphates is also an important factor. The fact that they were notsubstrates for thymidine phosphorylase and deoxynucleotidase-1, respectively, which are principal intracellular deoxynucleoside and deoxynucleotide catabolizing enzymes [8], is most likely a reason for the efficient accumulation of these metabolites in TK1 containing tumor cells.

Lesnikowski et al. reported the synthesis and biological evaluation of the first metallocarboranyl Thd analogues [44], which consist of eighteen boron atoms. The lipophilicities of this type of compounds were comparable with that of CDU. In preliminary experiment is has been demonstrated that

TK1 can accept Thd-N3 metallocarboranyl substitutions [45].

As mentioned above dCK is also a very attractive candidate for KMT and it is well known key enzyme in the activation of many anticancer nucleoside analogs [10,28,29]. However, although several 3CTA:s have been tested with highly active recombinant human dCK preparations, no clear-cut activity with these analogs was observed. The only boron containing nucleosides that show some activity with dCK were cyanoboranyl dCyd and dAdo, which demonstrated about 5-8% relative activity compared to dCyd [46].

Apparently the carboranyl cage may be too bulky to fit within the active site of dCK although this enzyme accepts many types of modified purine and pyrimidines and is known for its promiscuity [10,25]. So far a tethering out of site approach have not been successful with dCK but direct substitution with cyanobranyl groups may lead to synthesis of compounds that could be worth of further biological exploration. The mitochondrial deoxynucleoside kinases TK2 and dGK are not obvious candidates for the KMT principle in relation to BNCT since they are expressed in all cells and tissues although at different levels [10,46] and there is no obvious cut relation to proliferation or malignancies. These enzyme belong to the same enzyme family as dCK, and thus have a broad specificity but in this case they are either purine (dGK) or pyrimidine deoxynucleoside (TK2) selective. Since the 3CTAs are Thd analogues it was interesting to also test them with TK2, since CDU was shown to be a substrate for TK2 [9,35,46]. In general TK2 did not accept 3CTAs at levels above 5% of those with Thd, probably for the same reasons as mentioned for dCK. Several cyanoboranyl Thd and dCyd analogs tested with TK2 showed very low activity (2% or less) [46]. Recent preliminary experiments with 2′-O- and N3-metallocarboranyl deoxyuridine have demonstrated low but significant activity with TK2 [45].

To our knowledge, there are no studies using 3CTA monophosphates as substrates for the cellular deoxynucleoside monophosphate kinase enzymes. Therefore, it is at present not possible to predict if these type of nucleoside analogs can be further metabolized to the di or triphosphate levels and possible be incorporated into DNA. However, the monophosphate kinases are generally bilobal enzymes with their active sites buried in relatively deep clefts. Thus, it may not be very likely that bulky substituents, such as carboranes are accepted. However, the N3 position is not in direct interaction with active site residues in case of human TMPK [48]. Therefore, N3 substitutions could be also in this case, provided that the tethering is leading to an out of site location as in TK1. Experiments to design 3CTA analogs that could serve as substrates both for TK1 and subsequently for TMPK are warranted. The specificity of nucleoside diphosphate kinases for different base analogs is low but there is to our knowledge no information available regarding their activity with 3CTA

diphosphates. Most likely the complete synthesis of 3CTA triposphates is not a prerequisite for their BNCT efficacy, but in case of analogs with single boron substitutions incorporation into DNA could be a crucial step.

Conclusions

The experimental work summarized here provide evidence for that targeting DNA precursor enzymes, and in particular the proliferation associated enzyme thymidine kinase 1 with N3-carboranyl thymidine analogs, provides a mean to get selective accumulation of ^{10}B at sufficient levels for BNCT. The principles of placing the carboranyl cage outside the active site by an optimal tether as well as including hydrofilic substitutions to improve solubility seem to be essential for the success of this approach. So far targeting of other DNA precursor enzymes has not advanced to a similar extent but further work along these lines should be encouraged.

Acknowledgment

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METALLABORANE BUILDING BLOCKS AND THEIR USE IN DESINGN OF NEW NONPEPTIDOMETIC HIV- PROTEASE INHIBITORS

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Summary

This report summarizes some aspects of the chemistry of metal bis(dicarbollide) compounds functionalized by ammonium and oxonium groups and their use in design of inhibitors of HIV-PR, the key enzyme essential for the replication of the virus. The structure of the most efficient inhibitors consists from two cluster units combined together *via* an organic ammonium or amidic functions attached via several possible spacers. General aspects of use of boron cluster compounds for design of biologically active compounds are briefly discussed.

Introduction

Boron cluster compounds are currently used as inert carriers of boron to malignant tissue in Boron Neutron Capture Therapy (BNCT) [1-3]. A more important forward step with regards to potential applications is the use of substituted boron clusters as effective active substances in antiviral, antibacterial and anti-tumor therapies. Surprisingly enough, the potential use of boron clusters as pharmaceuticals beyond the BNCT area remains underestimated. Two reviews cover recent achievements in the field [4,5]. Typically, neutral o-carborane was used as a pharmacophore replacing phenyl rings in these systems. There are several, noteworthy reports describing carboranes as potential pharmaceutical, including e.g. antineoplastic and cytotoxic agents, estrogen agonists and antagonists, protein kinase C modulators and tranthyretin amyloidosis inhibitors [6-10].

In the field of metallaboranes, Fe(III) ferratricarbadecaboranyl salts [1-(η^5-C_5H_5)Fe-2-CH_3-2,3,4-$C_3B_7H_9$]AsF_6, as well as the neutral Fe(II) complex [1-(η^5-C_5H_5)Fe-2-CH_3-2,3,4-$C_3B_7H_9$], 4, have been tested as effective cytotoxic agents [11,12]. Vanada and niobatricarbadecaboranyl monohalide complexes proved to be potent cytotoxic agents against murine and human leukemia and lymphoma growth as well as HeLa suspended uterine carcinoma [13]. Small metallacarborane complexes containing C_2B_4 or C_2B_3 ligands and

Ta, Fe, Co, Mo, or W as central cations exhibited cytotoxic activity in murine and human tissue cultured cells [14].

For many years it has been our opinion, that boron clusters may potentially provide several advantageous features for the design of biologically active compounds. These include:

1) Most parent heteroboranes with *closo* (and 11-vertex *nido*) structures are chemically very stable, in general more than aromatic molecules and have been shown to have low toxicity and resistance to catabolism, where the respective data are available [5].

2) The cluster surface is composed of hydridic $B^{\delta+}$-$H^{\delta-}$ hydrogen atoms with no propensity to form classical hydrogen bonds (repulsion of water molecules). This causes strong hydrophobic or at least amphiphilic behaviour. It can be thus anticipated that a replacement of aromatic rings in some known therapeutics by a boron cage can enhance the biological activity in cases when hydrophobic and steric interactions play important role in the mechanism of a substrate binding. Several examples are known [5].

3) It can be assumed, that enzymatic systems, and especially these acting *via* degradation of phenyl rings, would be unlikely to split the boron cage composed of bonds absent in the natural systems [15]. Higher activity towards drug resistant mutated forms of various infectants can be thus anticipated. Several examples [16] support this assumption, such as the lack of decrease of activity for resistant forms of HIV as shown in preliminary results in this field [17-19].

4) There exist rich possibilities for tailoring their dipole moments or hydrogen binding sites of the molecule by introduction of heteroatoms, changing their geometric position, or spatial arrangement of the polar *exo*-skeletal groups, though there is only limited knowledge how to effect such substitutions in a geometrically pre-defined manner.

5) The acidity, basicity or nucleophilicity of each *exo*-skeletal groups (OH, SH, COOH, H_2N, etc.) can be significantly varied by attachment to either on boron or carbon atom of the cluster.

6) The protonated forms of the cluster anions behave as non-oxidizing inorganic superacids with no analogy in the area of organic chemistry [15]. On the other hand, they belong to a class named lowest

coordinating or weakly nucleophilic ions and this area is currently extensively studied [20-22]. The overall anionic charge is delocalized over the cluster and can be varied by inner cluster modifications. For example, formal replacement of two electron donor {BH} units in 12-vertex parent $[B_{12}H_{12}]^{2-}$ series by one or more main group moieties, e.g. {CH}or {P} (three electron donors) , or {PH}, {S} (four electron donors), produces $[CB_{11}H_{12}]^-$ $[PB_{11}H_{11}]^-$ anions or neutral $C_2B_{10}H_{12}$ carboranes, $SB_{11}H_{11}$ thiaboranes, etc.

7) Anions have high affinity to sites where a positive charge is located, i.e. interact strongly by non-covalent bonding with amino functionalities [15, 23] and peptides. This action has been recently also studied on a theoretical basis [24].

8) The charge density of the molecule is the main factors governing the different solubility characteristics of various boron species. Considering sodium salts, these of divalent anions are highly soluble in water, salts of univalent anions are soluble in medium polarity solvents (and can be extracted to them from water) and neutral compounds in lower polarity solvents only (aromatics, hexane) [15].

9) Another synthetic possibility to modulate the overall charge is the introduction of an *exo*-skeletal group e. g. $^1R^2R^3RN^+$, R_2S^+, R_3P^+ etc. This decreases cluster charge and introduces high dipole-moment. Conversely, the charge can be increased by cooperative action of several anions bound together on an organic platform.

10) This implies a broad possibility to modulate the overall charge of the molecule for each particular application. The surface area and charge of the cluster thus influence the hydrophobic or amphiphilic behaviour; hydrophobic interactions with receptors facilitate transfer across the cell membrane(1) whereas charge affects the solubility and the solution behaviour.

In this contribution we address the synthetic aspect of design and development of boron cluster compounds able to inhibit key enzyme of the Human Immunodeficiency Virus (HIV-1). HIV is the etiological agent of the Acquired Immuno Deficiency Syndrome (AIDS). The HIV replication cycle contains several virus-specific events that could be addressed by chemotherapeutic intervention. The compounds that are presently available as *anti*-HIV drugs are targeted either at the reverse transcriptase, the virus entry or the viral protease (PR). The current strategy for the treatment of HIV infection

is called Highly Active Antiretroviral Therapy (HAART) and is based on combination of at least three approved inhibitors from different families. The introduction of HAART has dramatically reduced mortality from AIDS-related diseases. Nevertheless multiple drug-resistant viral strains will finally emerge, followed by the progression to AIDS. Therefore, there persists a need for new anti-HIV-1 therapeutics less prone to resistance [25].

In our search for novel structural types of unconventional, versatile compounds that would inhibit HIV PR, a group of boron cluster compounds was identified as promising frameworks for a novel class of non-peptide PR inhibitors (PIs). Our main attention has been almost exclusively focused on metallacarboranes, especially on $[(1,2\text{-}C_2B_9H_{11})_2\text{-}3\text{-}Co(III)]^-$, 3-cobalt(III) bis(1,2-dicarbollide)(1^-) ion (**1**) [17,19,26].

The cobalt bis(dicarbollide) ion was synthesized by M. F. Hawthorne et all. more a four decades ago [27]. With time, this ion has attained a unique role in the chemistry of metallaboranes. This is due to its exceptional thermal, chemical and radiation stability, its single negative charge and its diamagnetic properties allow for an easy characterization of products based on this compound by ^{11}B, ^{1}H and ^{13}C NMR techniques. A review of its chemistry has appeared in the literature [28]. Also reviews on the use of the parent ion, its halogen derivatives and related functional molecules in extraction science have been published, last one in 2004 [29]. This is currently the most developed area for applications of the cobalt bis(dicarbollide) ion.

Despite many years of research, it is worth to mentioning here some basics facts which remain somewhat hidden in the previous reviews. The axial distances between the H(10)-H(10'), cluster positions in parent and substituted compounds are between 1.002 and 1.0036 nm. Calculated diameters of the circumscribed circle around the hydrogen atoms of the equatorial pentagonal ligand plane attached to the cobalt atom is close to 0.50 nm. The values are based on the data deposited in Cambridge Crystallographic Data Centre (CCDC). C-H sites of the ion are slightly acidic and can be deprotonated by BuLi. In solution, almost free rotation of ligand planes around the central atom is assumed. Three energy minimized structures have been calculated corresponding dihedral angles 180< 118< 36 degree (Relative Energies: 0.0, 1.6 and 10.6 kJ/mol)(30,31). The orientation of two couples of carbon atoms in the structure of the parent ion **1** is staggered (transoid) [28,31], but two other remaining rotamers can be found in many solid state structure of the substituted derivatives deposited in CCDC.

Surprisingly, little is known about solubility of the salts of **1** in water and their behaviour in aqueous solutions. The solubility of sparingly soluble caesium salt Cs**1** in water has been determined (620 μmol/L)(29), and solubility of some quarternary ammonium bases and amino acids has also been reported [23,29]. Calculated hydration free energy ΔG_{solv} of the cage is -17.9 Kcal/mol [24]. An extensive aggregation was reported very recently for dilute aqueous solutions of this and related ions upon ageing [32,33]. A driving force for this is apparently a tendency to limit contacts of the hydrophobic surface of the ion composed from hydridic B-$H^{\delta-}$ bonds with the surrounding water molecules. It is obvious, that hydrated cations should be incorporated in such aggregates to preserve condition of electroneutrality. The role of the cation present in experimentally studied has not yet been addressed, though this has been reflected in molecular modelling studies of the behaviour of **1** at the interface of water and an immiscible solvent [34,35].

Along with electrophilic substitutions, the main substitution mechanism entering into play with the cobalt bis(dicarbollide)(1-) anion (**1**) has been defined as the so called "Electrophile Induced Nucleophilic Substitution" EINS-1. In this path a Lewis acid activator abstracts the most hydridic hydrogen B(8)-H (or also B(8')-H) position(s) and opens up vacancy(ies) for the insertion of a nucleophile [28].

Parent metal bis(dicarbollide) anions as HIV-PR inhibitors

Even the parent unsubstituted ion **1** has been proved to act as an efficient HIV inhibitor exhibiting a moderate activity. On the other hand combinations of several clusters bonded *via* an organic central unit have, in general, several orders higher efficiency [19,26]. A short survey on synthesis of the systems consisting of several boron clusters is presented below along with some remarks addressing their efficiency. More details on inhibitors evaluation and solution behaviour will be given by other Czech Groups cooperating on this subject.

The parent cobalt bis(dicarbolide) anion has been proved to inhibit the HIV-PR at micromolar level and acts in a competitive manner [26]. Several other known isostructural and isoelectronic metallaborane ions [28,36] containing eleven vertex ligands were tested. These include $[(1,2\text{-}C_2B_9H_{11})_2\text{-}3\text{-}Fe(III)]^-$ (**2**), $[(1,2\text{-}C_2B_9H_{11})_2\text{-}3\text{-}Ni(III)]^-$ (**3**), $[(1,2\text{-}C_2B_9H_{11})_2\text{-}3\text{-}Cr(III)]^-$ (**4**) $[(1,7\text{-}C_2B_9H_{11})_2\text{-}2\text{-}Co]^-$ (**5**) and $[(1\text{-}SB_{10}H_{10})_2\text{-}2\text{-}Co(III)]^-$ (**6**) [37] (see Fig. 1). The ions exhibited similar inhibition activity in *in vitro* tests, but last two provided a quite different, noncopetitive and uncompetitive mechanism of inhibition.

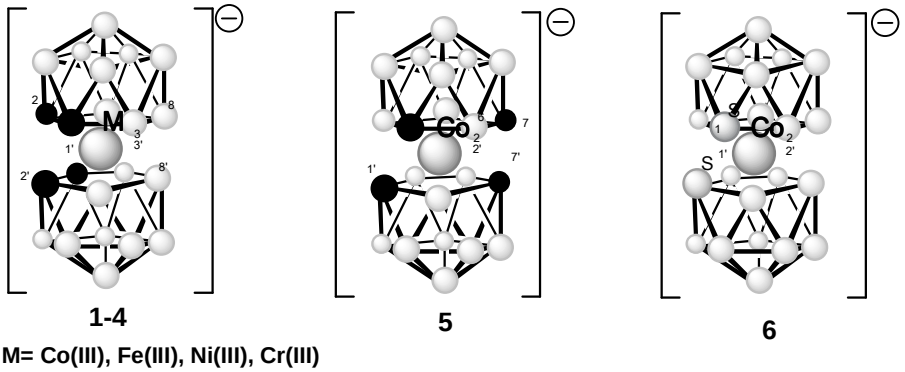


Figure 1. Schematic structures of the metal bis dicarbollides $[(1,2\text{-}C_2B_9H_{11})_2\text{-}3\text{-}M]^-$ (M(III) = Co, Fe, Ni, Cr), (**1-4**) $[(1,7\text{-}C_2B_9H_{11})_2\text{-}2\text{-}Co]^-$ (**5**) and $[(1\text{-}SB_{10}H_{10})_2\text{-}2\text{-}Co(III)]^-$ (**6**).

With the current knowledge it seems difficult to suggest a reason for this difference and the answer may be complex. Either the higher acidity of carborane CH groups in **1** affecting specific bonding in the HIV-PR complex, or the hydrophobicity of the ions resulting in different solution behaviour may play a role. Also a combination of the two factors could be responsible for the observed difference. Nevertheless, the influence of hydrophobicity seems more supported, since the substitutions by hydrophobic groups at B(8), B(8') skeletal positions (phenyl of phenylene), leads to noncompetive mechanism of inhibition, and the substitution of B(8,8') atoms by iodine in $[(8,8'\text{-}I_2\text{-}1,2\text{-}C_2B_9H_{11})_2\text{-}3\text{-}Co(III)]^-$ leads to uncompetitive action. The acidity functions of CH groups in these systems are unknown, but at least all of these ions can provide similar kind of interactions as parent ion **1**. Methyl or hexyl substitution at carbon sites of the ion **1** led to an increase of efficiency but also reverses the competitive mechanism to noncompetive. If we consider the HPLC capacity factor (k') values at Reverse Phase C8 column as a semi quantitative measure of hydrophobicity, the order for parent ions is (k' values in parentheses) **1** (3.7) < **2** (4.1) ≤ **3** (4.2)< **4** (4.5) << **5** (8.6) << **6** (12.6). Indeed, there is noticeable increase in hydrophobicity for the last two members of the series.

Substitutions at B(8) (B(8')) sites by polar groups such as -OH, >O, $-O\text{-}(CH_2CH_2)_2OH$, $-OP(O)(OH)_2$ led in turn, to a decrease of the inhibitor's efficiency. Complete or severe loss of efficiency is observed for zwitterionic derivatives bearing alkyl ammonium groups.

Several smaller cage carboranes and *nido* anions and their derivatives were also tested, but these compounds in general proved much less efficient,

similarly to the neutral half sandwich $[(\eta^5(C_5H_5)\text{-}3\text{-}Co(III)(1,2\text{-}C_2B_9H_{11})]^0$ or the organometallic ferrocenium and cobaltocenium cations tested for comparison. Tests showed (with few exceptions) that exclusively ionic species act as the effective HIV-PR inhibitors.

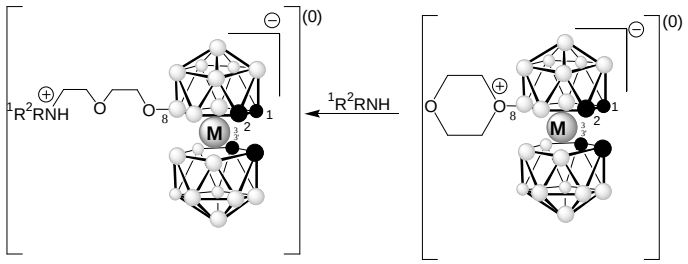
Inhibitors composed from several cluster units

Construction of oligomeric materials based on covalent bonding of charge neutral boron clusters, especially these with linear shape, has attracted increasing attention during past years. A well explored area is that of small carboranes and metallaboranes that allow for easy *in situ* generation of stable multidecker array [38,39,40-42] or *exo*-skeletal bonding to various organic substrates in C-C and B-C manners [43]. The area of icosahedral boron clusters is comparatively understudied. The availability of building blocks for linear such constructions has been discussed in a review [44]. The first stable rod-like molecules based on C-C and C-B bonding to *p*-carborane units have been reported in 90's [45-47]. Recently, linear double cluster constructions based on 12-vertex ferratricarbollide were reported [48]. Chemistry of neutral carborane macrocycles based on bonding of neutral carboranes *via* mercury into cyclic rings has been developed [49]. A relatively rich area is that of designed molecules composed of neutral carboranes linked by organic spacer groups [50-56]. These include also cycles based on *m*-carborane, molecules bonded *via* thiaalkylgroups, 2,6-pyridyl and aryl groups, and compounds designed for purposes of BNCT and as temperature resistant oligomers [57-59].

Comparatively underexplored is the area of ionic compounds. Double cage carborane- dodeca- borate(2-) and bis(dodecaborate)(4-) ions bonded *via* a chain have been prepared [60]. Interesting is the area of nanosized carborane closomers assembled on $B_{12}(OH)_{12}^{2-}$ core [61,62]. We have reported on the bonding of several cobalt bis(dicarbollide) clusters together *via* an organic linker to supramolecular platforms such as calix[4]arenes or resorc[4]arenes in a geometrically pre-defined manner [63] followed by reports on the introduction of the cluster anions and metal binding groups to the platform [64]. Recently, several papers appeared in the literature on synthesis of porphyrines bearing several cobalt bis(dicarbollide) clusters designed for BNCT and photodynamic therapy and as HIV-PR inhibitors [65].

The most efficient lead structures prepared to date are based on combination of two cluster units bonded together *via* an organic central part comprising ammonium or amidic function [26,66]. Several tens of new ionic constructions of this type have been prepared and tested over the last years. Such compounds are easily available in high yield from a reliable toolkit of ammonium derivatives in combination with Plesek's original synthetic concept [67] of ring opening of the B(8) dioxane-cobalt bis(dicarbollide) zwitterion [68] (**9**) or closely related compounds.

The library of available ammonium derivatives used as construction blocks comprises several derivatives prepared some time ago by this Group. These include the bridged [8,8'<H_2N-(1,2-$C_2B_9H_{11}$)$_2$-3-Co]$^-$ [69] (**10**) and the asymmetric compounds [4,8'<H_2N-(1,2-$C_2B_9H_{11}$)$_2$-3-Co]$^-$ [70] and [6,6'<H_2N-[(1,7-$C_2B_9H_{11}$)$_2$-2-Co]$^-$ [71] (**11, 12**) and an almost endless number of combinations easily generated by ring opening reactions of **9** or other compounds of this type [72,73] (see below) by any primary or secondary amine of choice (also boron cage amines such as [1 –NH_3-$B_{12}H_{11}$]$^-$ [74] (**13**) or [1 –NH_3-$CB_{11}H_{11}$] [75] (**14**) were used in molecular design). The intermediates produced by this approach thus usually have the ammonium group attached to the cage by a diethyleneglycol spacer (**15**).

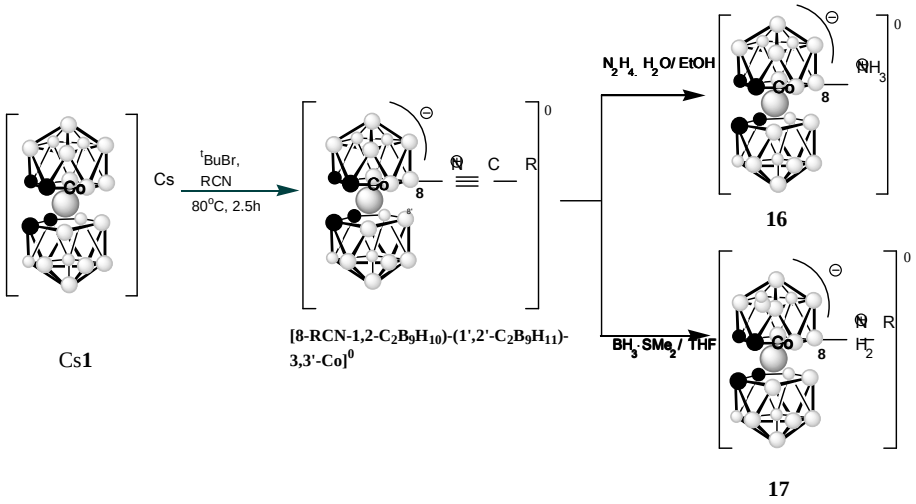


15 **M= Co(III), Fe(III), Cr(III) (9, 18, 19)**

Scheme 1. Ring cleavage of the 8-dioxane derivatives of metal bis(dicarbollides) generating various ammonio derivatives **15** for construction of HIV-PR inhibitors.

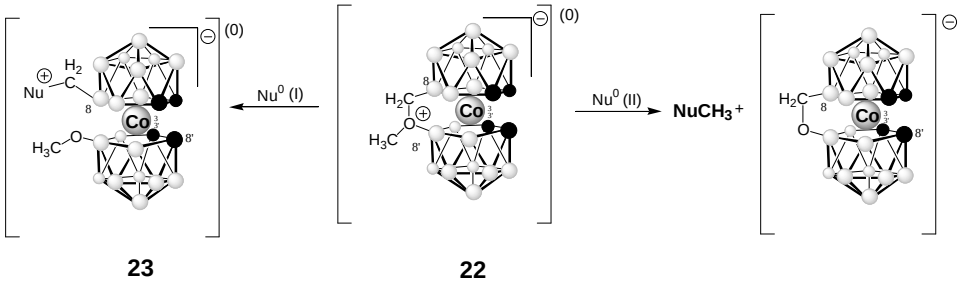
To explore better the structure-performance relationship, recently a search was performed for B(8) ammonium derivative of ion **1**. We found, that the reaction of *t*-butyl bromide serves as potent activator for (B8) substitution of the cage by N-atom of acetonitrile (or other nitriles) which results in smooth formation of [8-RCN-1,2-$C_2B_9H_{10}$)(1',2'-$C_2B_9H_{11}$)-3,3'-Co]^0 derivative. These compounds further serves as a versatile precursors for the preparation in the anion **1** series of long time anticipated ammonium derivative and a variety of synthetically useful functional groups. Thus hydrazinolysis of the acetonitrile function gives the zwitterionic ammonium derivative [(8-NH_3-1,2-$C_2B_9H_{10}$)(1',2'-$C_2B_9H_{11}$)-3,3'-Co] (**16**) in excellent yields. Reductions using $BH_3.SMe_2$ provide instead moderate yields of zwitterionic 8-alkylammonium derivatives [(8-RNH_2-1,2-$C_2B_9H_{10}$)(1',2'-$C_2B_9H_{11}$)-3,3'-Co] (**17**).

The triple bond of the nitrile functions is prone to an easy addition of amines or alcoholates. Former reaction thus results in high yield formation of 8-alkylamidine derivatives whereas 8-alkylamide-**1** are obtained in the later case [76]. Some compounds from these series were tested alone as HIV-PR inhibitors, but with rather discouraging results.



Scheme 2. Synthetic ways to B(8) ammonio derivatives of the cobalt bis(dicarbollide).

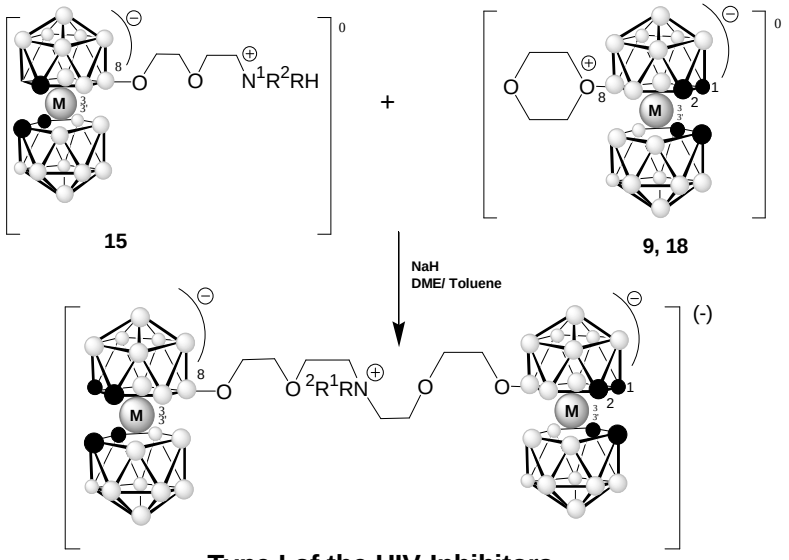
The second reagent in the main reaction scheme is represented by oxonium oxygen containing compounds. The former and still the most versatile compound from the metallaborane series, 8-dioxane-**1** was first reported by Plesek and Franken in 1997 (**9**) [68]. Reactions of oxonium ring compounds were very recently reviewed by other authors [73]. Prompted by this and other emerging biochemical applications, we have recently focused on the synthesis of similar compounds based on other metal bis(dicarbollides). Compounds from iron(III) (77) and chromium(III) bis(dicarbollide) [78] and bis(thiaundecaborate) [37] sandwich series (**18**-**20**) were successfully prepared and characterized by NMR and M. S. Some of their simple derivatives resulting from ring opening reactions were characterized by X-ray crystallography. Similar, very reactive building block (**21**), containing tetrahydrofurane ring could be obtained from bulkier double decker "dicobalt bis(dicarbollide) canastide" ion [79] using BF_3OEt_2 catalysis [80]. Most of these zwitterions and derived amines have been already employed as alternatives to the compound **9** in the inhibitor's design.



23 **22**

Scheme 3. Reaction paths of bridge [8,8'μ-$CH_3O^+CH_2$(1,2-$C_2B_9H_{10}$)$_2$-3,3'-Co] zwitterion (**22**) with amines as nucleophiles.

A slightly different ring system is represented by compound [8,8'μ-$CH_3O^+CH_2$(1,2-$C_2B_9H_{10}$)$_2$-3,3'-Co] (**22**). This compounds results as product from the acid catalyzed reactions of Cs**1** with formaldehyde, along with the known bridge zwitterion [8,8'μ-CH_3O<(1,2-$C_2B_9H_{10}$)$_2$-3,3'-Co] present as a side product. The oxonium ring of the compound is readily opened by a primary or secondary amine as a nucleophile producing [(8-$^1R^2$RNH-CH_2-1,2-$C_2B_9H_{10}$)-(8'CH_3O-1',2'-$C_2B_9H_{10}$)-3,3'] cage substitutions (**23**). Exceptions exists for sterically hindered or strongly basic amines, when this compound serves as a methylating agent, when the CH_3 group sitting at the bridge is abstracted, preserving the ring arrangement of the substituent B(8)-CH_2-O-B(8') in the resulting anionic product.



Type I of the HIV-Inhibitors

Scheme 4. Synthetic approach providing Type I of double cluster HIV-PR inhibitors.

The synthetic amalgamation of these two kinds of building blocks (exemplified in Scheme 4), is quite simple, efficient, and almost quantitative. The ring of compounds bearing an oxonium oxygen atom (**9, 18**-**22**) is cleaved by compounds containing the ammonium functionality (**10-17, 23**) producing a double cluster molecule. Symmetric or asymmetric structural motifs are available as shown in Scheme 4 and Fig. 2. If a boron building block with primary amine function is used for the ring opening, three cluster molecules can be obtained in one additional step.

Another approach was made *via* the reactions of ammonium derivatives with dicarboxylic acid dichlorides. This produces the respective diamides. These diamides were primary designed for extraction science, but proved effective also as HIV-PR inhibitors.

The three lead structures obtained by these means are shown in Fig. 2. It should be noted, whereas the first two types are monoanionic due to protonation of the amine moiety by influence of the metal bis(dicarbollide) acid behaviour, the third type is two minus charged. Only the first type is competitive, the remaining two types represent noncopetitive or uncompetitive inhibitors, depending on particular substitution of the nitrogen atoms.

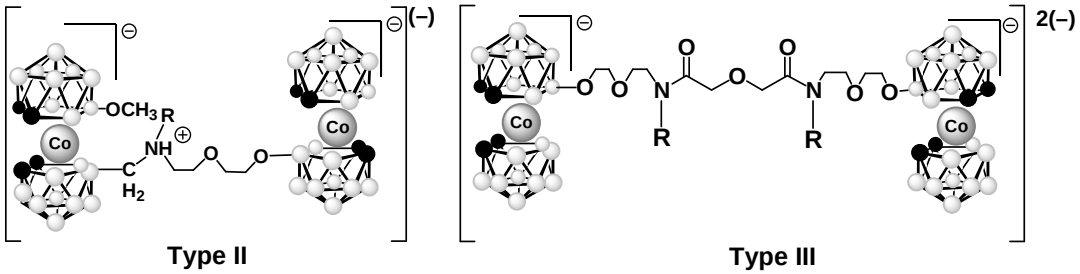


Figure 2. Schematic structures of the asymmetric (Type II) and amidic (Type III) inhibitors of HIV-PR. Type II is based on combinations of building block **23** with oxonium compounds **9, 18, 19**. Type III was synthesized from ammonio derivatives **10, 15, 17.**

Conclusions

Our previous and ongoing research in the chemistry of reactive metallaborane building blocks enabled the easy and efficient synthesis of several types of double cluster compounds which can serve as the potent HIV protease inhibitors. The preliminary results from *in vitro* tests carried out at IOCB AS ČR and tests *in vivo* in tissue cells performed at the University of Heidelberg (Group of Prof. H.-G. Kraeusslich) indicated high efficiency of several lead structure types as inhibitors of HIV-1 protease, especially in respect to resistant HIV virus strains [18,19]. These compounds show specific

inhibition activity and good selectivity [26]. Convincing results from the biochemical studies will be presented during BioBor Meeting by speakers from the cooperating Teams. Further structural design and synthesis of boron cluster HIV-1 protease inhibitors, carried out under close collaboration of scientists from IOCB and IIC, is currently in progress.

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ROLES FOR POLYHEDRAL BORANES AND CARBORANES IN NANO AND MOLECULAR MEDICINE: PREVENTION OF AMYLOID DISEASE BY TRANSTHYRETIN COMPLEXATION WITH CARBORANE DERIVATIVES LACKING CYCLOOXYGENASE INHIBITION

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Summary

Transthyretin, a homotetrameric transport protein found primarily in the blood and cerebrospinal fluid, is the putative causative agent in a variety of amyloid diseases. Kinetic stabilization, through small-molecule complexation, of the native tetrameric state of transthyretin may impart a protective effect through prevention of the dissociation that leads to amyloid fibril formation. However, many of the compounds known to impart this stabilization are, or are structurally similar to, non-steroidal anti-inflammatory drugs (NSAID). Consequently, such compounds exhibit deleterious concomitant inhibition of cyclooxygenase (COX) enzymes. Through judicious application of carboranes as skeletal motifs in analogs of these NSAIDs, a compound, 1-carboxylic acid-7-[3-fluorophenyl]-dicarba-*closo*-1,7-dodecaborane, was synthesized that showed effectively no COX-1 or COX-2 inhibition at a concentration more than an order of magnitude larger than the concentration at which TTR dissociation is nearly completely inhibited.

Key words: Carborane, thyroxine, transthyretin, amyloid, cyclooxygenase

Introduction

The three isomeric dicarba-*closo*-dodecaboranes (carboranes); *closo*-1,2-$C_2B_{10}H_{12}$, *closo*-1,7-$C_2B_{10}H_{12}$ and *closo*-1,12-$C_2B_{10}H_{12}$, commonly known as *ortho*, *meta* and *para*-carborane respectively, are icosahedral carbon-containing boron clusters that share approximately the same volume as a rotated phenyl ring and may be described as three-dimensional analogs of aromatic hydrocarbons [1]. Much of the extensive chemistry of carboranes has been utilized to further their exploration as agents of high boron-content for use in Boron Neutron Capture Therapy (BNCT), thus providing a wealth of information indicating the biocompatibility and resistance to catabolism of a variety of carborane-supported structures [2]. Recently, the use of carboranes

as novel pharmacophores has garnered increasing interest, primarily due to their extraordinary characteristic properties; such as resistance to catabolism, kinetic inertness to reagents and elevated hydrophobicity [3]. These varied properties have facilitated the application of the carborane moiety in an exceptionally diverse variety of biological targets including HIV protease inhibitors [4,5], insect neuropeptides [6] and α-human thrombin inhibition [7] as well as analogs of the anti-estrogen tamoxifen [8], the controversial drug thalidomide [9] and the antifolate trimethoprim [10]. Endo and co-workers harnessed the lipophilicity of the carborane moiety to great effect in the synthesis of potent retinoid antagonists [11] and estrogen agonists [12]. Concurrent investigations illustrated that the hydrophobicity of carborane derivatives may be fine-tuned through the choice of position of substitution on the carborane cage, which is a facile prospect given the ease of regioselective derivation of the both the *C*- and *B*-vertices of the cage [13]. In an effort to expand the medicinal chemistry of carboranes, we have endeavored to identify further biological targets where the unique properties of carboranes may prove to be beneficial.

Transthyretin (TTR), previously known as thyroxin-binding prealbumin, is a homotetrameric protein comprised of four identical 14-kDa, 127-amino acid subunits exhibiting 2,2,2 symmetry in an extended *beta*-sheet conformation [14,15]. Human TTR is encoded on chromosome 18 and is highly conserved (over 80% sequence homology) in mammals [16]. TTR does not cross the blood-brain barrier and is therefore made mainly in the liver and choroid plexus which produce the supplies found in human plasma (0.2 mg/mL, 3.6 μM of tetramer) and the cerebrospinal fluid (CSF) (0.02 mg/mL, 0.36 μM of the tetramer), respectively [14,15]. Minor amounts of TTR are synthesized within the retina and meninges [16]. In both the blood and CSF, TTR binds and transports thyroxine (T_4), a hormone, in two hourglass-shaped binding sites defined by the dimer-dimer interface (Figure 1) and also forms a complex with retinol binding protein which in turn transports vitamin A [15-20].

In 1978, Costa *et al.* identified TTR as the major constituent of amyloid fibrils associated with familial amyloid polyneuropathy (FAP) [21]. TTR has subsequently been isolated from the fibrils associated with a varied assortment of amyloid diseases. Deposition of wild-type TTR (WT-TTR) has been implicated as the causative agent in senile systemic amyloidosis (SSA), a cardiac malady that affects approximately 25% of the population over 80 years old to some degree [22].

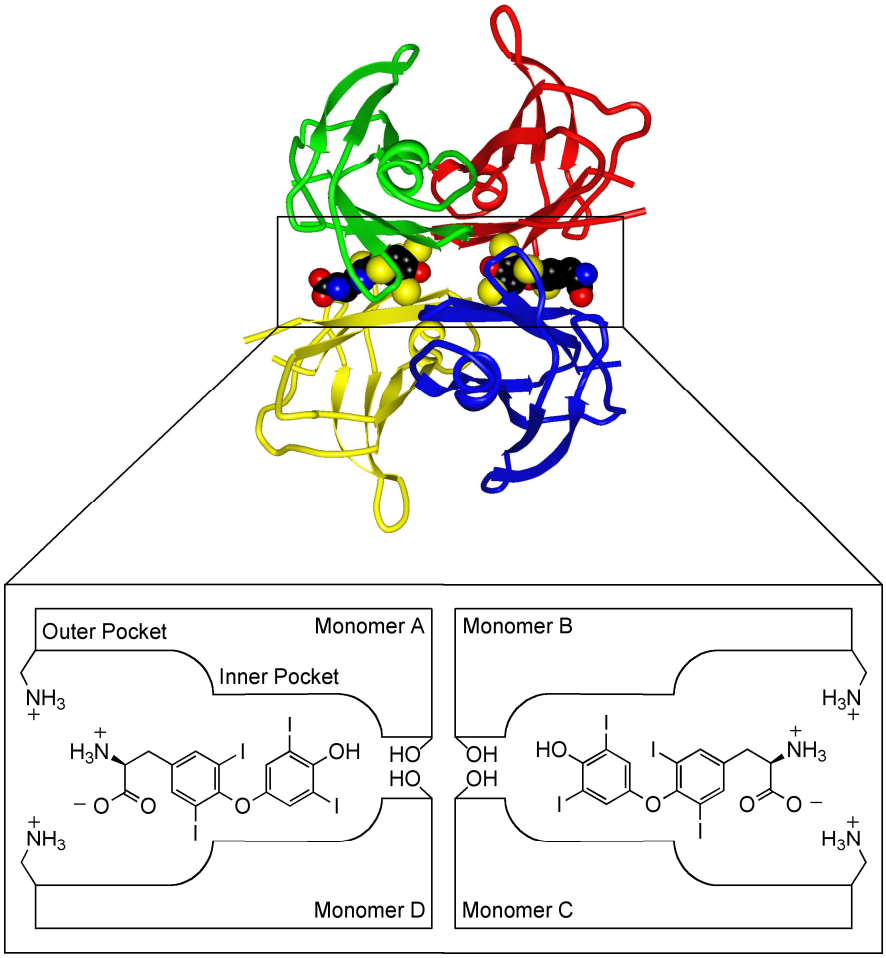
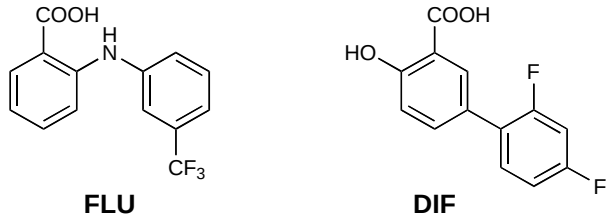


Figure 1. Ribbon diagram of human WT-TTR co-crystallized with T_4 (PDB ID: 1ICT) shown perpendicular to the symmetric T_4 binding site with an expanded **s**chematic representation of the symmetrical TTR-T_4 binding channel formed at the dimer-dimer interface of four TTR monomers. Electrostatic interactions between the polar head group of T_4 and K15 and K15' occur at the mouth of the spacious outer pocket while perturbations of the protein structure within the inner pocket facilitate hydrogen bonding primarily between S117 and S117' across the binding channel, resulting in tetramer stabilization.

Accumulation of one of over 100 identified TTR variants leads to the hereditary diseases of familial amyloid cardiomyopathy (FAC), central nervous system selective amyloidosis (CNSA) and the previously mentioned FAP (23). FAC is the most widely distributed of these, with about 4% of African-Americans carrying the mutation that causes a predisposition to the disorder

[24]. Currently, treatment of these hereditary diseases centers on liver transplantation, in which a liver producing WT-TTR is substituted for the FAP variant-producing organ. While surgical intervention may provide a temporary reprieve for the patient, cardiac amyloidosis proceeds in many cases due to the continued accumulation of WT-TTR amyloid fibrils [25]. Coupled with the shortage of available livers, the risks associated with transplantation and the fact that transplantation can impede neither WT-TTR expression nor TTR synthesis within the eye or the choroid plexus, a pharmacological solution appears to be an attractive alternative.

Studies have indicated that the mechanism of TTR amyloid fibril formation proceeds through tetramer dissociation to a monomeric intermediate that subsequently aggregates to form the putative pathogenic protofibrils and finally amyloid fibrils [26-28]. However, the native conformation of TTR can be kinetically stabilized *in vitro* by T_4 and structurally similar derivatives thereof [29]. Given that less than 0.5% of the two thyroxine binding sites within TTR are occupied *in vivo*, investigations have focused on small molecule inhibitors which stabilized tetrameric TTR without undesirable hormonal activity [30]. This research has been successful in identifying a wide variety of structurally diverse compounds that impart kinetic stabilization to tetrameric TTR [31]. However, many of the most promising compounds are known nonsteriodal anti-inflammatory drugs (NSAIDs), such as flufenamic acid and diflunisal (Figure 2) or structurally related species.



FLU **DIF**

Figure 2. Two NSAIDs, flufenamic acid (**FLU**) and diflunisal (**DIF**) that are potent inhibitors of TTR fibril formation.

NSAIDs derive their pharmacological effect from the inhibition of cyclooxygenase (prostaglandin endoperoxide synthase or COX) enzymes [32]. Of the three known isozymes of COX in the human body, COX-1, COX-2 and COX-3, both COX-1 and COX-2 are well defined. Inhibition of COX-1 may lead to adverse gastrointestinal events, a situation that is estimated to result in 103,000 hospitalizations and 16,500 deaths in the United States each year [33], while COX-2 has been prominently associated with an increased risk of cardiovascular events [34]. Clearly, given the long-term nature of TTR stabilization therapy, a drug candidate with a minimum of COX activity would be preferable.

The hydrophobic binding channels in TTR appear to be ideally suited for the utilization of carboranes as a skeletal core. Crystal structures of TTR indicate that the funnel shaped T4 binding site can be generalized into a spacious outer binding pocket large enough to bind sterically bulky substituents and a smaller inner pocket, as shown in Figure 1 [35]. It was hypothesized that the three-dimensional carborane structure would fill the outer pocket in order to maximizing hydrophobic interactions. It was further hypothesized that COX activity could be reduced by this steric bulk as well as the inability of the carborane moiety to participate in π-π stacking utilized by many COX inhibitors, but unnecessary for inhibition of TTR dissociation [36]. This report describes the synthesis of a group of carborane-based compounds and their efficacy as potent inhibitors of TTR dissociation and subsequent fibril formation. We further screen those compounds recognized as promising inhibitors of TTR amyloid formation and identify a lead compound that also lacks any significant cyclooxygenase inhibitor activity.

Results and Discussion

Using the knowledge gained from previous structure-activity studies, carborane-containing analogs of known inhibitors of TTR fibril formation inhibitors were synthesized (Figure 3). The *in vitro* efficacy of these TTR stabilizers was rapidly assessed via acid-mediated denaturation [37]. This assay employs TTR at the physiological concentration (3.6 μM) incubated with a varying amount of potential stabilizer and then subjected to partially denaturing conditions at pH 4.4 for 72 hours. Fibril formation is then quantified by measuring the optical density (OD) of the solution at the end of the time course and the results reported as percent fibril formation (% *ff*), with total inhibition of fibril formation set equal to 0% *ff* and TTR in the absence of inhibitor defined as 100% *ff*. In essence, a lower % *ff* indicates a more potent inhibitor of TTR fibril formation. These carborane-based analogs were shown to inhibit TTR fibril formation in an identical fashion to a known TTR stabilizer, flufenamic acid, in a dose-dependent assay (Figure 3), with **1** and **5** yielding 22±2 and 15±5 %*ff* respectively at an inhibitor concentration of 3.6 μM, while flufenamic acid produced 14 ± 4% *ff* under equivalent conditions.

Compounds **1** and **2** were then screened for cyclooxygenase activity, initially employing a commercially available COX-1 (ovine) and COX-2 (human recombinant) enzyme immunoassay according to the manufacturer's instructions (Cayman Chemical). The compounds were screened at 100 μM, a concentration several times that required to nearly completely inhibit TTR fibril formation. Compound **1** exhibited some COX inhibition, with 6.0±5.9 and 57±10 % initial activity for COX-1 and COX-2 respectively.

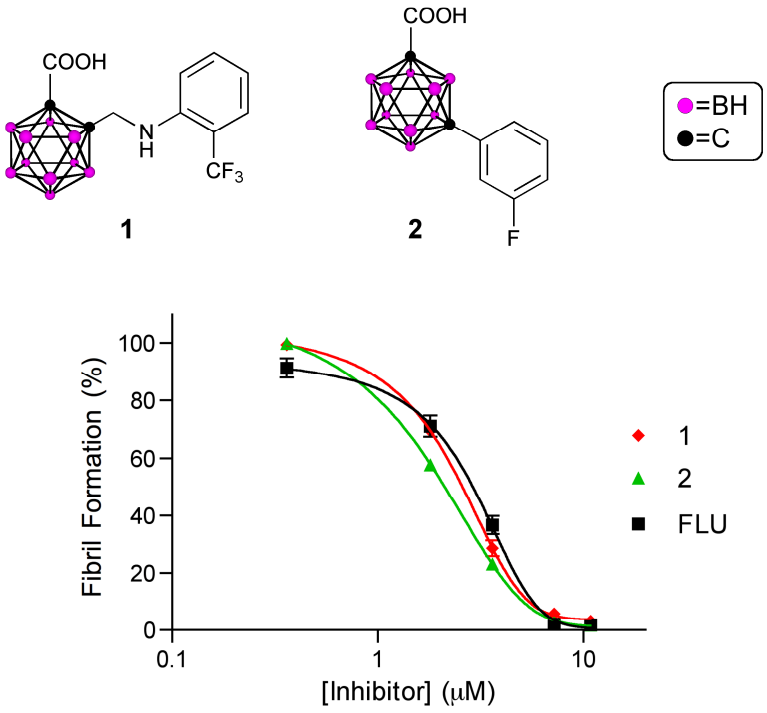


Figure 3. Carborane-based analogs of flufenamic acid (**1**) and diflunisal (**2**) screened in **a s**tagnant TTR (3.6 μM) fibril formation assay (pH 4.4) at varying concentrations over 72 hours.

However, compound **2** showed virtually no COX-1 (93±13 % initial activity) or COX-2 (94±16 % initial activity) in this assay. This difference may be due to the increased flexibility and length of **1** with respect to **2**, allowing it to slip into the stericly encumbered COX binding channel. In order to directly compare the COX activity of **2** relative to flufenamic acid, a full COX inhibition curve was run with **2** and flufenamic acid (Figure 4) using a commercially available colorimetric COX screening assay. Inhibition assays for flufenamic acid and **2** were run for ovine COX-1 (Figure 4A) and COX-2 (Figure 4B) at various concentrations. Compound **2** proved to be a virtually imperceptible inhibitor of both COX-1 (IC50> 200 μM) and COX-2 (IC50 > 100 μM) isoforms at a concentration more than an order of magnitude larger than the concentration necessary for **2** to perform exceptionally well with regard to the inhibition of TTR amyloid formation, making it an ideal lead compound for further study [38].

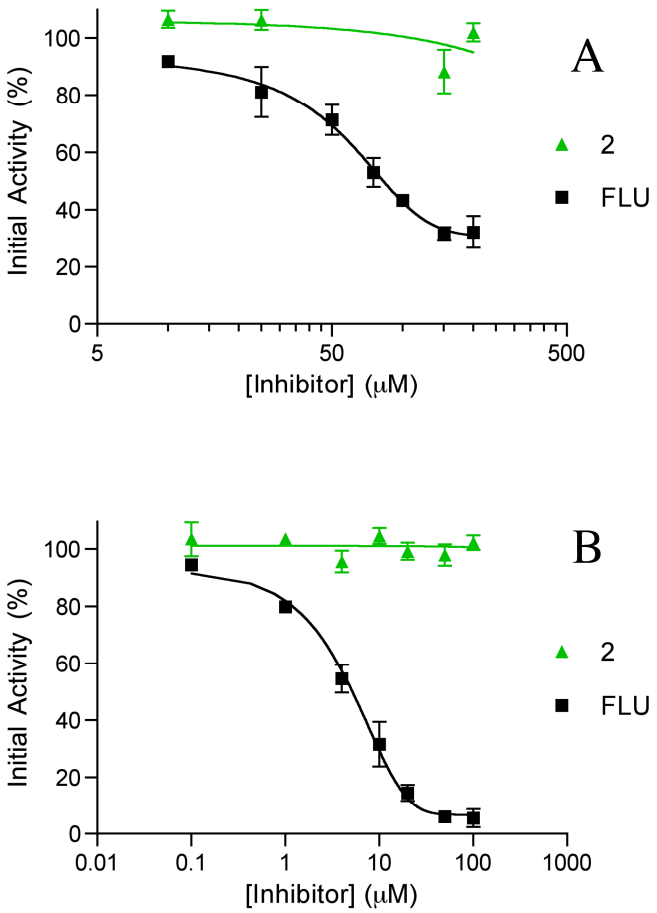


Figure 4. Colorimetric COX-1 (A) and COX-2 (B) screening assay employing compound **1** and flufenamic acid (**FLU**).

Conclusion

The *in vitro* identification of a new transthyretin (TTR) stabilizer that does not inhibit COX enzyme activity has been described here. This favorable set of biological characteristics is apparently made possible by the use of an icosahedral carborane cage as a scaffold structure. This change enhances lipophilicity and eliminates the π-π stacking possible in known TTR stabilizers that also show undesired COX enzyme inhibition. Further work with biologicals amenable to similar structural surrogation is in progress.

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IMITATION AND MODIFICATION OF BIOLOGICALLY RELEVANT OR ACTIVE MOLECULES VIA INTEGRATION OF CARBABORANE CLUSTERS

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Summary

High and selective accumulation in tumour cells is one important requirement for a BNCT agent. One way of increasing the tumour selectivity of BNCT agents may be the use of carbaborane-containing bisphosphonates. As some simple carbaboranylbisphosphonates already exhibit high tumour selectivity, we are attempting to improve the BNCT potential by using glycosyl esters of phosphonic acids.

Key words: BNCT, carbaboranes, phosphonates, tumour selectivity.

Introduction

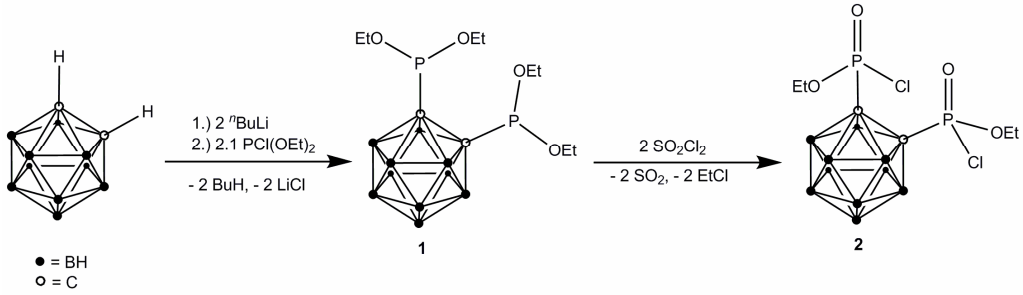
To date, the treatment of malign tumours is always accompanied by extremely negative side effects. One potentially useful approach for the selective destruction of tumour cell is boron neutron capture therapy (BNCT), a powerful form of radiotherapy involving preferential incorporation of ^{10}B-containing compounds into tumour cells, followed by irradiation of the tumour by thermal neutrons [1]. The high-energy fission products which are formed on absorption of a neutron allow selective destruction of the tumour cells without affecting the surrounding healthy tissue. High and selective accumulation in tumour cells is one important requirement for a BNCT agent [1-3]. For successful treatment, a concentration of 30 µg ^{10}B per gramme tumour must be achieved. The main problem to date is the availability of boron compounds which exhibit the necessary high selectivity, water solubility and low toxicity in high concentrations.

Carbaboranylphosphonates are known as biologically active compounds. When the carbaboranyl group is incorporated into the ester moiety of a phosphorus acid, these compounds show high anticholinesterase activity [4]. Compounds in which the carbaborane is attached to the phosphorus acid through a sulfur or selenium atom show bactericidal activity [5]. Interestingly, some simple carbaboranylbisphosphonates exhibit high tumour selectivity and can be used in the treatment of calcifying tumours [6]. Oligomeric phosphate diesters which contain *closo*- or *nido*-carbaboranes show high accumulation in tumour

tissue in BALB/c mice bearing EMT6 tumours [7]. However, comprehensive biological assessments of boron-containing phosphonates as potential tumour-targeting BNCT agents are still rare [8].
We have therefore devised efficient syntheses for novel boron compounds which provide a combined tumour-targeting system: The use of phosphonato groups as phosphate mimics and galactosyl groups for binding to lectine at the surface of a tumour cell [9].

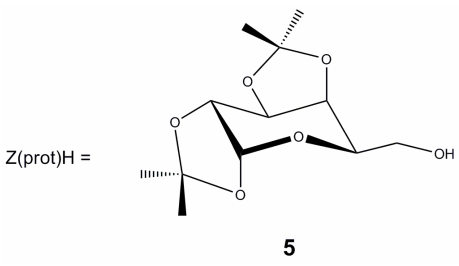
Results

The synthesis of sugar-containing carbaboranylbisphosphonates was attempted starting from carbaboranylbis(chlorophosphonates) and a protected sugar (with one hydroxyl group still available). Thus, carbaboranylbis(chlorophosphonate) (**2**) was obtained from **1** by chlorination with sulfuryl chloride (Scheme 1).



Scheme 1. Synthesis of **2**.

However, we were unable to convert compound **2** to the target compound by reaction with 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**3**) under different reaction conditions; in all cases, only decomposition was observed (Scheme 2). As phosphorus(V) substituents on *ortho*-carbaborane exhibit only low reactivity, the more reactive phosphorus(III) derivatives were employed. However, no reaction occurred between the dialkylamidohalophosphonito-substituted *ortho*-carbaboranes 1,2-$\{P(NR_2)X\}_2C_2B_{10}H_{10}$ (R = Me, ^{i}Pr, X = Cl, Br) and isopropylidene-protected galactose **5**. Employing the less electron-withdrawing *meta*-carbaborane instead of the *closo*-carbaborane was also unsuccessful.



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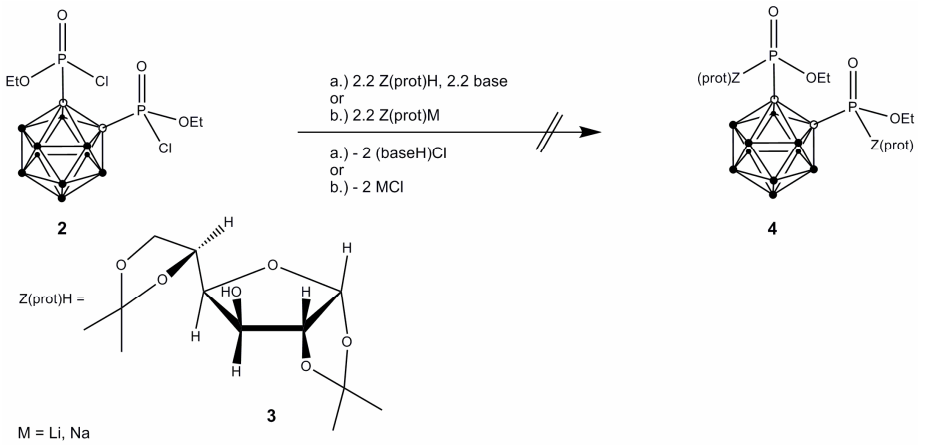
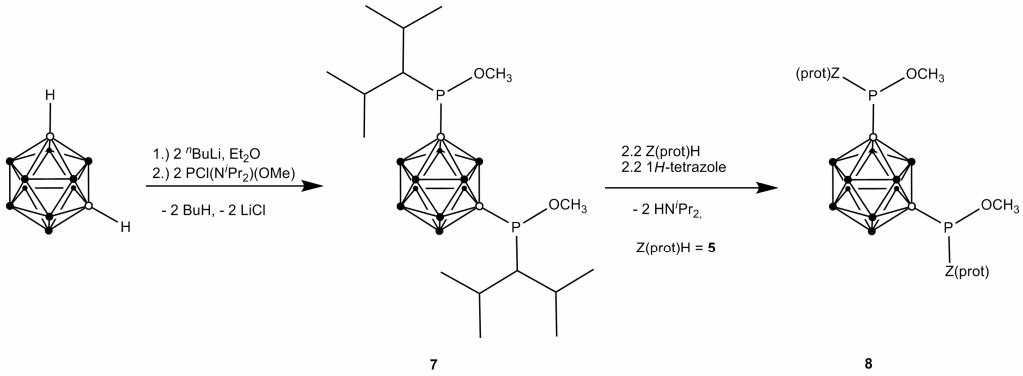


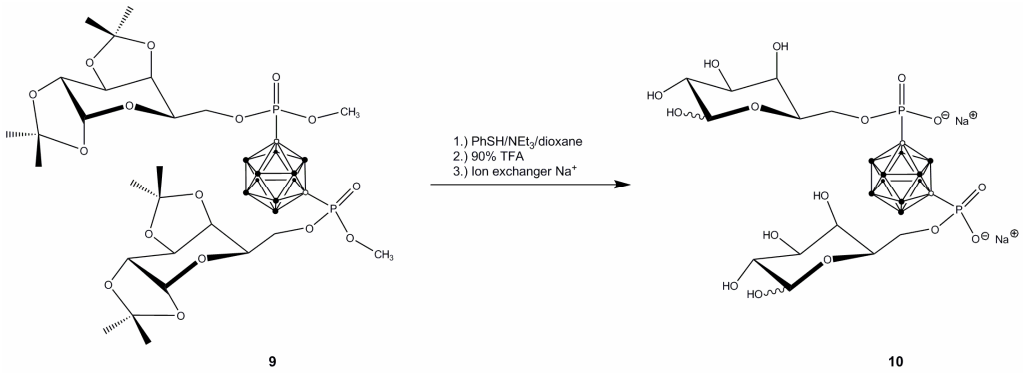
Scheme 2. Reaction of **2** with the sugar derivative **3** led to decomposition.

Attempts to employ the activation of a P–N bond by an acidic catalyst, which is well known from oligonucleotide synthesis, to replace the amido group in 1,2-{P(N^{i}Pr$_2$)(OMe)}$_2$C$_2$B$_{10}$H$_{10}$ (**6**) with the sugar **5** was not successful when 1*H*-tetrazole was employed as catalyst. A reaction occured at 140 °C, however, with cleavage of the P–C$_{Carbaboran}$ bond. We have therefore employed the analogous *meta*-carbaborane **7**, which could be galactosylated with catalysis by 1*H*-tetrazole with **5** (Scheme 3).



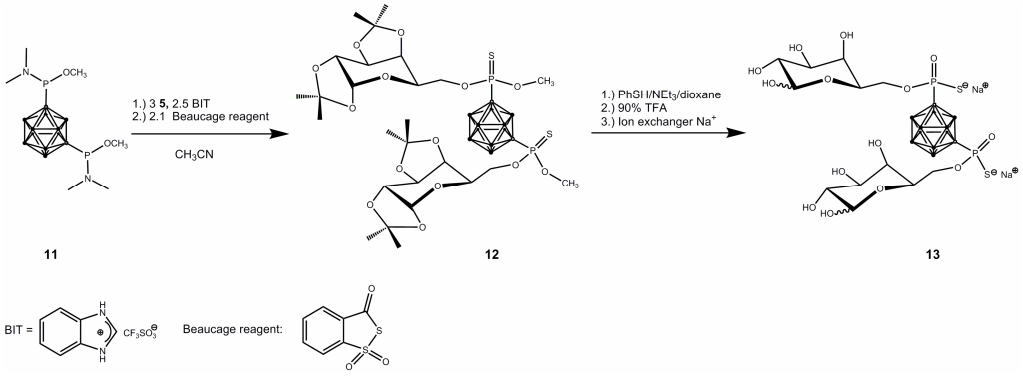
Scheme 3. Galactosylation of the *meta*-carbaborane derivative **7**.

The synthesis of **8** could be improved by using benzimidazolium triflate (BIT) as catalyst under microwave conditions and by replacing the N^{i}Pr$_2$ group with the less sterically demanding NMe$_2$ group. Compound **8** was oxidised with *tert*-butylhydroperoxide (TBHP) *in situ* to the bisphosphonate **9** which was obtained as four diastereomers. Deprotection of the galactosyl and phosphonato groups with trifluoroacetic acid and conversion into the sodium salt gave the water-soluble compound **10** (Scheme 4).



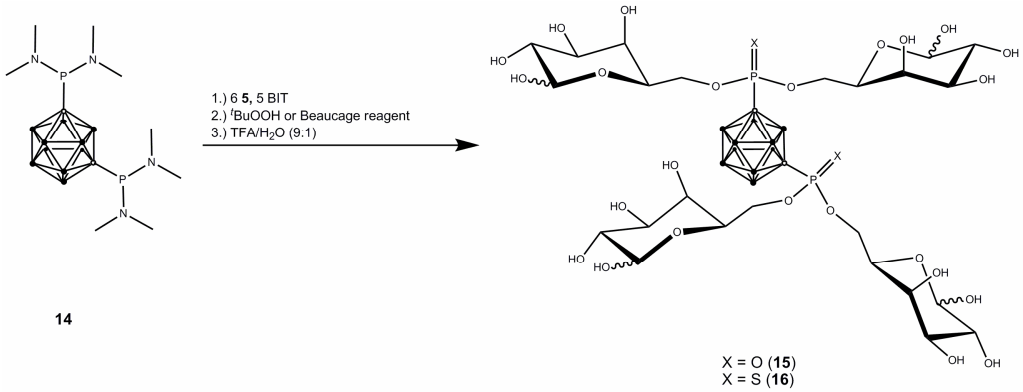
Scheme 4. Deprotection of the galactosyl and phosphonato groups in **9**.

For improved *in vivo* stability towards phosphatases and phosphonate esterases the corresponding bisphosphonothioate **13** was pepared accordingly by this method using 3*H*-1,2-benzodithiol-3-one-1,1-dioxide, the so-called BEAUCAGE reagent, instead of TBHP (Scheme 5).



Scheme 5. Synthesis of the bisphosphonothioate **13** from **11**.

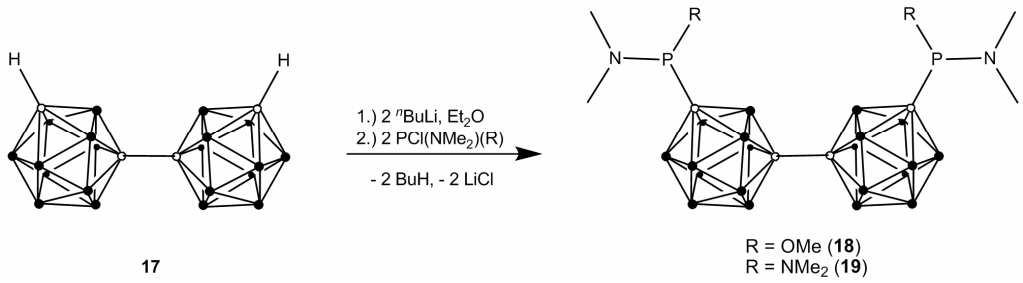
The synthesis of fully galactose substituted carbaboranylbisphosphonates **15** and **16** was achieved starting from **14** and **5** by employing the same protocol as described above (Scheme 6).



Scheme 6. Synthesis of tetrakisgalactosyl substituted carbaboranylbisphosphonic acid.

The biological activity of **10**, **13**, **15** and **16** was tested on tumour cells of the line HeLa by employing the resazurin assay. Compounds **10** and **13** do not show cytotoxicity up to a concentration of 20 mM and are thus far less toxic than the boron compounds which are presently employed in BNCT, i.e., sodium mercaptoundecahydrododecaborane (BSH, IC_{50} 3.9 mM), rendering them interesting candidates for BNCT. Compounds **15** and **16** show higher toxicity (EC_{50} ca. 29 mM (**15**), 14.0 mM (**16**)), which is, however, still lower than that of BSH. This can be attributed to the higher lypophilicity of these compounds.

Compounds with higher boron contents were obtained via copper-mediated C–C coupling of monolithiated *meta*-carbaborane. Functionalisation of the C–H groups of the obtained bis(*meta*-carbaborane) (**17**) with $PR(NMe_2)$ groups (R = NMe_2 (**18**), OMe (**19**)) (Scheme 7) followed by galactosylation, oxidation or sulfurisation, deprotection and conversion to the sodium salt gave the galactosylphosphonato-substituted bis(*meta*-carbaborane)s **20, 21, 22, 23** (Fig. 1).



Scheme 7. Synthesis of bisphosphonito substituted bis(*meta*-carbaborane) derivatives.

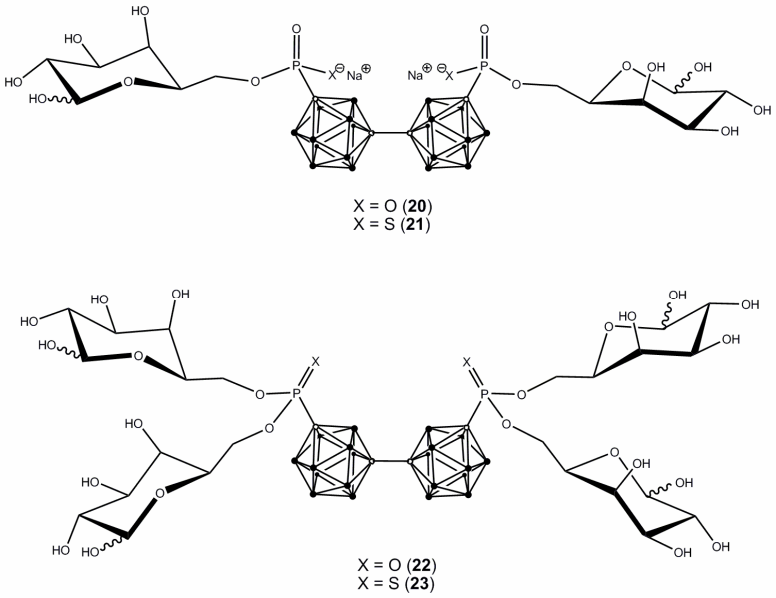


Figure 1. Galactosylphosphonato substituted bis(*meta*-carbaborane)s.

The presence of the second lypophilic carbaborane cluster increases the cytotoxicity. Thus, the bisphosphonate **20** exhibits an EC_{50} value of 19.5 mM and the bisphosphonothioate **21** a value of 2.1 mM (Fig. 2). Compounds **22** and **23** have lower water solubility than the other derivatives resulting in low EC_{50} values on HeLa cells (480.9 µM (**22**), 174.9 µM (**23**)). Therefore, compounds **21, 22, 23** are unsuitable for application in BNCT.

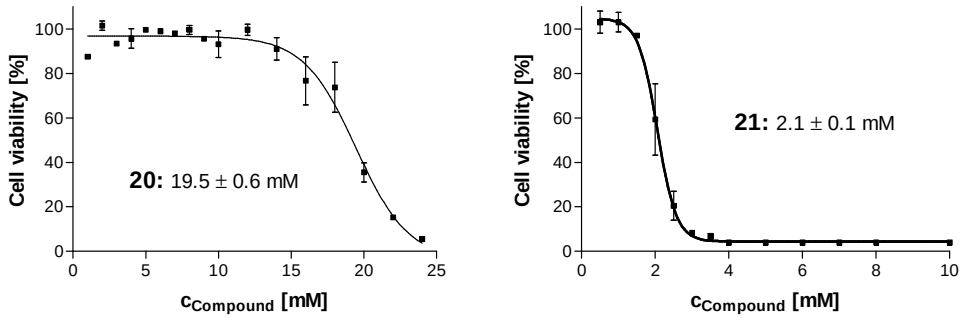
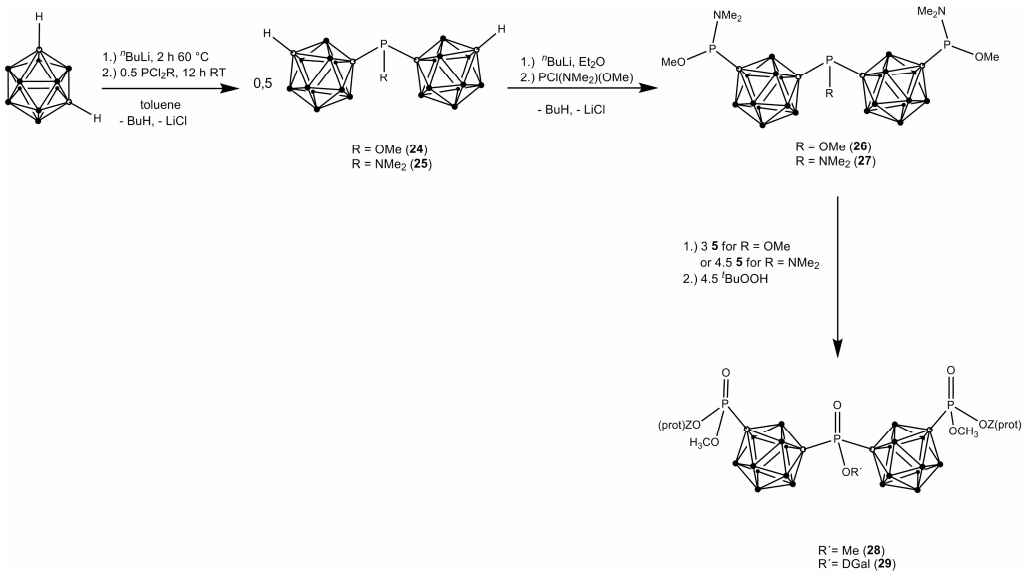


Figure 2. Cell toxicity data for bisphosphonate **20** and bisphosphonothioate **21**.

To increase the boron contents but to also improve the water solubility of biscarbaboranyl derivatives, syntheses of compounds in which the two carbaboranyl clusters are bridged by a hydrophilic phosphinate group were attempted. Monolithiation of the *meta*-carbaborane at high temperatures proved necessary to produce the biscarbaboranylphosphinites **24** and **25**. Conversion of the terminal CH groups to $P(NMe_2)(OMe)$ is readily achieved, but galactosylation of 1,1´-{7,7´-$[P(NMe_2)(OMe)]_2[C_2B_{10}H_{10}]_2$}P(OMe) (**26**) followed by oxidation gave the desired product **28** only in low yield, while 1,1´-{7,7´-$[P(NMe_2)(OMe)]_2[C_2B_{10}H_{10}]_2$}$P(NMe_2)$ (**27**) can be galactosylated only at the terminal P atoms but not at the bridging P atom (Scheme 8). An improved synthesis for **29** remains to be developed.



Scheme 8. Synthesis of P-bridged bis(*meta*-carbaborane) derivatives.

Compounds **10**, **13**, **15**, **16** and **20** exhibit low cytotoxicity and are thus suitable for studies concerning tumour selectivity. Preliminary studies were carried out in collaboration with Prof. GABEL, Universität Bremen.

The bisphosphonothioates **13** and **16** were chosen as representative examples to study the *in vivo* toxicity in Swiss mice. It was shown that compound **16** (dosage 100 mg/kg boron) results in toxic side effects, while compound **13** was well tolerated and thus employed in further boron distribution studies. For these studies four female BALB/c mice with a CRL tumour were treated intraperitoneal with a dosage of 100 mg/kg boron. The mice were euthanised after certain periods of time and frozen thin sections were made, which will now be studied to obtain the boron distribution.

Conclusion

We have developed a suitable synthesis employing the phosphoramidite methode to connect *meta*-carbaboranylbisphosphonites with the 6´-OH group of isopropylidene-protected galactose, followed by oxidation or sulfurisation to give the corresponding bisphosphonates. Deprotection yielded water-soluble compounds. The corresponding disodium salts exhibit especially low cytotoxicity. The fully galactosyl-substituted derivatives are, however, less water soluble and show higher cytotoxicity. Therefore, phosphinato-bridged bis(*meta*-carbaborane) derivatives seem to be more suitable also with respect to increasing the boron contents. In general, the bisphosphonothioate derivatives exhibit higher cytotoxicity than the bisphosphonato derivatives. Preliminary results on the *in vivo* toxicity and biodistribution of two compounds in mice were obtained.

Future studies will focus on different glycosides and their respective linkage with the phosphorus atom. Thus, glucose, mannose or disaccharides such as lactose, aminosugars such as galactosamine and different connectivities, e.g., via the anomeric position, will be employed, and their biological activity will be evaluated.

Acknowledgement

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BEYOND PYRIMIDINE NUCLEOSIDES AND CARBORANES - NEW NUCLEOSIDE/BORON CLUSTER CONJUGATES AND THEIR APPLICATIONS

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Summary

Several general methods for the synthesis of purine and pyrimidine nucleosides modified with carborane clusters and metallacarborane complexes is discussed. They include: 1) attachment of carborane modification at 2' position of nucleoside *via* formacetal linkage formation, 2) tethering of the metallacarborane group at nucleobase part of the nucleoside *via* dioxane ring opening in oxonium metallacarborane derivatives and 3) "click chemistry" approach based on Huisgen 1,3-dipolar cycloaddition. Proposed methodologies extend the range of nucleoside/borane cluster conjugates available and open new areas for their applications.

Key words: nucleosides, carboranes, metallacarboranes, conjugates, molecular probes, redox label.

Introduction

Due to their essential role in virtually all cellular processes nucleosides are one of the most important small biomolecules. They are basic building blocks of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), plays many important functions as themselves, in phosphorylated form (nucleotides) or in conjugation with other biomolecules [1].

Because their crucial role in many metabolic pathways and interactions with other biomolecules nucleosides, their derivatives and analogues are widely used as chemotherapeutics, mainly as antiviral or anticancer agents [2,3]. A number of nucleosides are currently on the market for the treatment of various diseases, many others will be marketed soon.

Practical applications of nucleosides and theirs analogues range beyond chemotherapy - a variety of labeled nucleosides are used as tools in molecular biology in structure-function relationship study, are used in synthesis of nucleic acids probes, and are finding applications in such fields as PCR, microarrays and biosensors technology as a part of the modified DNA-oligomers.

Nucleoside is more than the sum of carbohydrate and heterocycle, and interaction with DNA is not the only mechanism for biological activity of nucleosides. A nucleoside may be an ideal transport system for a heterocycle, selectivity with nucleosides may be obtained just by making use of natural uptake systems, or a nucleoside analogue may have a beneficial effect on the immune system, which contributes to its biological activity.

The chemistry of nucleosides and nucleic acids continues to be a rapidly developing field of study [4,5]. Countless nucleosides, nucleotides and nucleic acids analogues were synthesized, and strange sugar-containing antibiotics, such as neplanocins and oxetanosin were discovered.

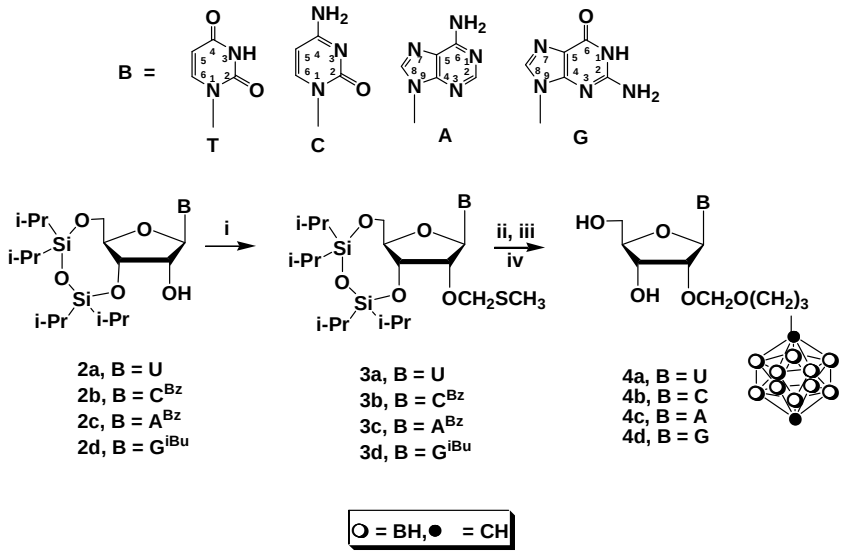
Boron containing nucleosides were originally designed as potential boron carriers for boron neutron capture therapy (BNCT) of tumors [6,7]. As boron rich donor 1,2-dicarba-*closo*-dodecaboranes ($C_2B_{10}H_{12}$, *ortho*-carborane) cage was most frequently utilized [8]. The rational for the synthesis and study of these modifications is that such compounds may be selectively accumulated in rapidly multiplying tumor cells, and following their conversion to the corresponding nucleotide, trapped within the cell or ideally, incorporated into nuclear DNA of tumors [9,10].

However, the chemistry of boron modified nucleosides has implications further than BNCT [11,12]. The carboranyl clusters are used as modifying entities for oligonucleotides potentially useful as antisense agents for antisense oligonucleotide therapy (AOT) and as molecular probes for molecular diagnostics based on hybridization technology [13,14], as a lipophilic pharmacophors [12,15,16], electrochemical [17,18] and infrared labels [19], and others [20]. Factors limiting applications of born cluster/nucleoside conjugates include among others: 1) lack of variety among the boron clusters that are accessible to the medicinal chemists – by far most approaches utilized the *ortho*-carborane cage, 2) confinement of the nucleoside part of the conjugate to the pyrimidine nucleosides series – with very few exceptions only thymidine or uridine derivatives modified with boron unit were synthesized and studied till now, 3) neglecting of the potential of the metallacarboranes as modifying units for nucleosides. Approaches to surpass these limitations in particular directions will be discussed.

General method for the modification of nucleosides at 2'-position with *para*-carborane cluster.

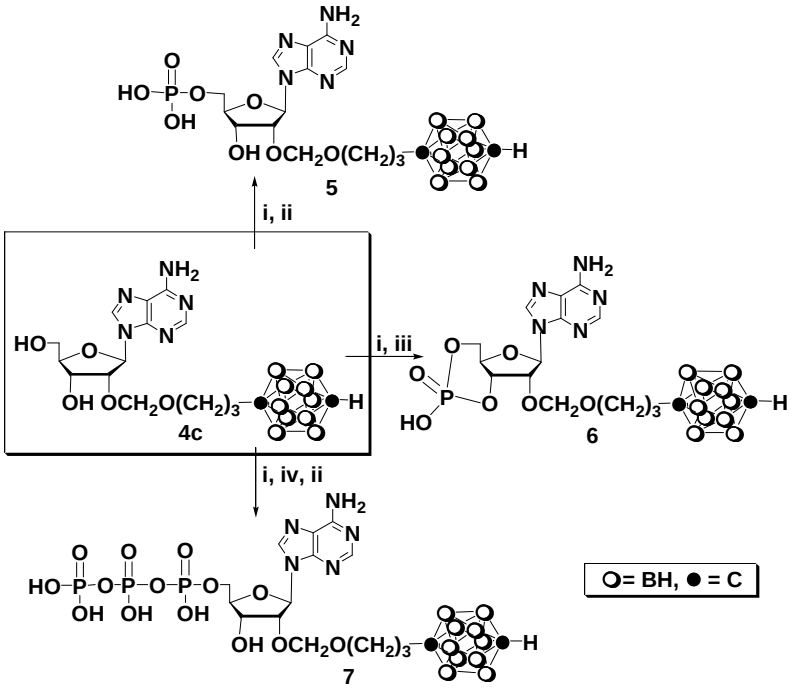
Most of carborane modified nucleosides described so far belong to pyrimidine series [21]. Despite the fact that purine nucleosides such as adenosine and guanosine play an important role in cellular metabolism this class of carborane modified nucleosides focused less attention. For example, synthesis of a carborane-containing purine nucleoside inosine was attempted, but the 2-*ortho*-carboranyl-inosine precursor could not be deprotected [22].

Another type of boron containing purine as well as pyrimidine nucleosides consists not a cluster but a cyanoborane group as a single boron atom donor [23]. The successful approach [24,25] to the synthesis of 2'-*O*-[(*para*-carboran-1-yl)propyleneoxymethyl] derivatives of all four canonical nucleosides **4a-d** is based on the formacetal linkage formation described by Matteucci [26] and modified by Sawada and Ito [27]. It utilizes nucleophilic substitution of the activated methylthiomethyl group in fully protected 3',5'-*O*,*O*-(tetraisopropyldisiloxane-1,3-diyl)-2'-*O*-methylthiomethylnucleosides (**3a-d**) with a suitable alcohol bearing carborane cage (Scheme 1).



Scheme 1. i. DMSO/AcOH/Ac_2O, ii. TBABr/1-(3-hydroxypropyl)-*para*-carborane (1)/ $CuBr_2$ in CH_2Cl_2, iii. TBAF/THF, iv. 2M NH_3aq in CH_3CN.

The key intermediates **3a-d** were obtained in the reaction of *N*-protected 3',5'-*O*,*O*-(tetraisopropyldisiloxane-1,3-diyl)nucleosides **2a-d** with DMSO in a mixture of acetic acid/acetic anhydride. The *N*-protected 3',5'-*O*,*O*-(tetraisopropyldisiloxane-1,3-diyl)nucleosides **2a-d** are easily available and can be prepared according to the literature procedure from suitable ribonucleosides: uridine (U), cytidine (C), adenosine (A) and guanosine (G) giving high yield. Target compounds **4a-d** were obtained from **3a-d** in a three-step procedure without isolation and purification of the intermediate products. First compounds **3a-d** were reacted with 1-(3-hydroxypropyl)-*para*-carborane (**1**) [28] yielding fully protected 3',5'-*O*,*O*-TIPDSi-2'-*O*-[(*para*-carboran-1-yl)propyleneoxymethyl]nucleosides, then the disiloxane protection was removed using a solution of TBAF in THF yielding *N*-protected 2'-*O*-[(*para* carboran-1-yl)propyleneoxymethyl]nucleoside. The acyl protections were removed with concentrated aqueous ammonia solution providing **4a-d**.



Scheme 2. i. $POCl_3/P(O)(OEt)_3$, ii. 1M TEAB, iii. 0.08M KOH in H_2O/CH_3CN, iv. $H_4P_2O_7/Bu_3N$.

It is worthy to point out that the proposed approach offers a route to nucleoside conjugates modified with different types of carborane cages or other functional groups as long as suitable alcohol terminated with the intended functional group is available.

Our method provides an opening for the synthesis and study of nucleic acids modified with carborane clusters at designed locations [13,14] and of other biologically important derivatives of nucleosides. For example adenosine derivative **4c** was used for synthesis of important adenosine phosphates, AMP, cAMP and ATP modified with *para***-**carborane cluster (Scheme 2) [29]. The adenosine phosphates modified with *para*-carborane are characterized by increased stability in human blood plasma and more than three orders of magnitude higher lipophilicity than that of the unmodified phosphates. ATP analog bearing *para*-carborane cluster is not a *Taq* polymerase substrate and most probably not the polymerase inhibitor. These properties may have clinical implications.

Metallacarborane nucleoside conjugates.

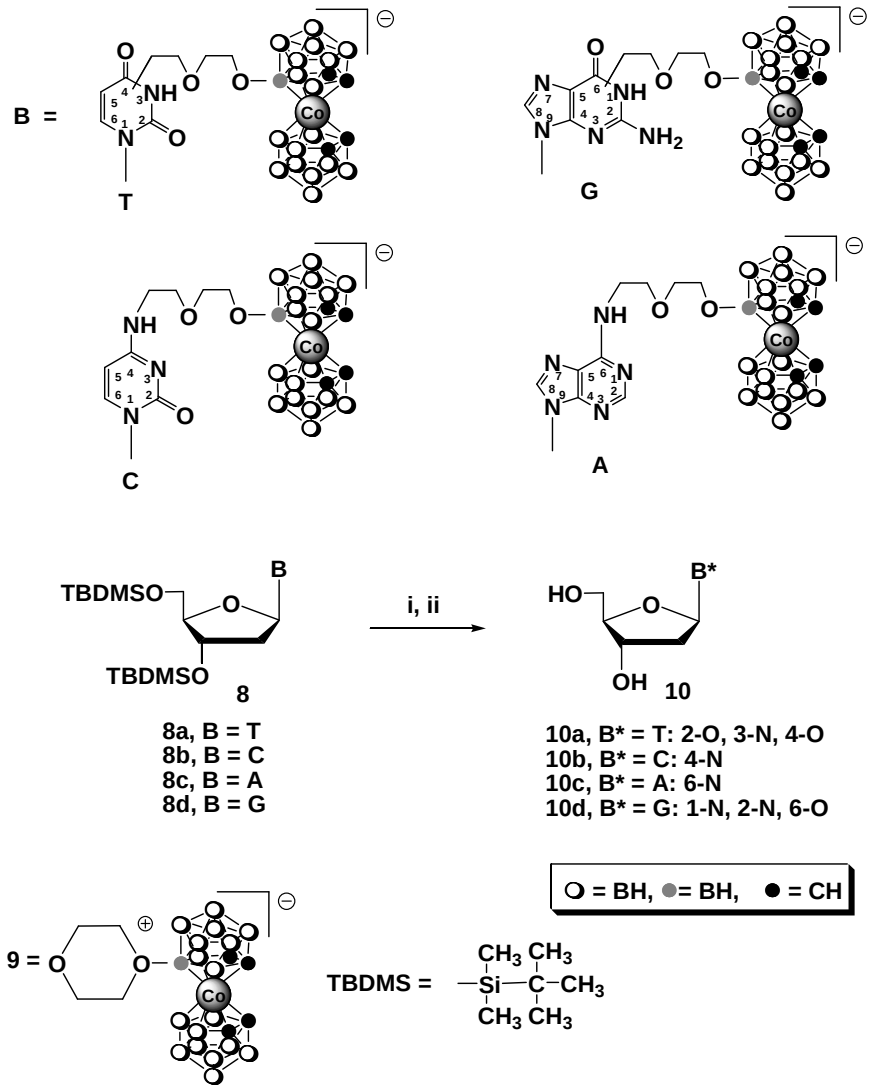
Metallacarboranes, after long neglecting of the study of their biological properties, are presently pursued by many investigators for their anti-viral [30] and anti-tumor activities [31,32]. It is feasible that conjugates of nucleosides and metallacarboranes could exhibit useful biological characteristics. Another advantage of metallacarborane-nucleosides is their application as versatile synthons for synthesis of metal bearing DNA-oligomers for various applications.

Metallacarboranes can function as electrochemical and photo luminescent labels for nucleic acids, infrared labels, radioactive metal isotope carriers, active centers of DNA-directed artificial chemical nucleases, metal bearing components in construction of probes for DNA-mediated electron transfer, and others [14].

We proposed a general approach to the synthesis of nucleoside conjugates, derivatives of thymidine (T), 2'-*O*-deoxycytidine (dC), 2'-*O*-deoxyadenosine (dA) and 2'-*O*-deoxyguanosine (dG), containing metallacarborane complex. Metallcarborane-nucleoside derivatives have been prepared in the reaction of ring opening in dioxane-metallacarborane adduct by base activated 3',5'-protected nucleoside [33].

The ring opening in the cyclic ether attached to [bis(1,2-dicarbollido)-3-cobalt(-1)]ate ion by simple nucleophiles was described earlier [32,34,35]. Recently a synthesis of porphyrin containing [bis(1,2-dicarbollido)-3-cobalt(-1)]ate ion was described, but application of the above method for complex biological molecules have not been persuaded [36].

The target nucleoside-metallacarborane conjugates were obtained in a simple, three-step procedure. First, 5'- and 3'-hydroxyl functions of nucleosides **1a-d** were protected. For that purpose *tert*-butyldimethylsilyl protection was used for both hydroxyl groups. The 3',5'-*O*,*O*-di(*tert*-butyldimethylsilyl)-2'-*O*-deoxynucleosides **8a-d** are easily available and can be prepared according to the literature procedure from suitable nucleosides giving high yield. In the second step, each of 3',5'-protected nucleosides **8a-d** was activated with an excess of sodium hydride then treated with dioxane-metallacarborane adduct **9** in anhydrous toluene as reaction medium. In the third step, the *tert*-butyldimethylsilyl protections were removed with tetrabutylammonium fluoride providing metallacarborane-nucleoside conjugates **10a-d** containing 5-[3-cobalt bis(1,2-dicarbollide)-8-yl]-3-oxa-pentoxy- modification at different locations within the nucleic acid base (Scheme 3).



Scheme 3. i. NaH/toluene, **9**, 70°C; ii, TBAF/THF.

The present approach can provide a route to nucleoside conjugates modified with metallacarboranes bearing different metals and different types of carborane cages as long as suitable adducts of the cyclic ether and boron cluster is available.

Indeed, nucleoside/metallacarborane conjugates bearing iron, chromium or rhenium have been also obtained. Synthesis and some properties of these novel compounds will be discussed in more details in one of the following chapters. Also nucleoside conjugates containing *closo*-dodecaborate anion $(B_{12}H_{12})^-$ have been prepared *via* ring opening in suitable cyclic oxonium adduct analogously to the method described above [37].

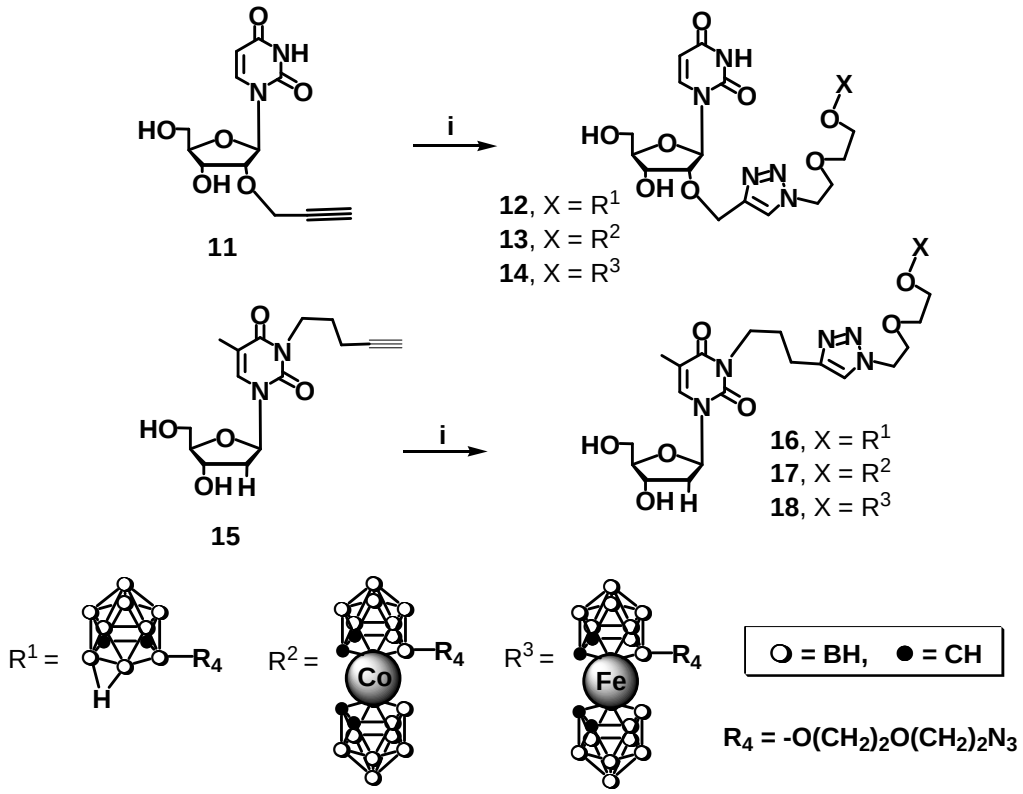
Availability of such methodology makes possible studies of a broad spectrum of nucleoside conjugates bearing metal and incorporation of metal centers into DNA oligomers at designed locations. It shows also a versatility of nucleophilic ring opening in dioxane-metallacarborane adducts as a new approach for the metallacarborane attachment to biological molecules [33].

"Click-chemistry"- A general and versatile method for modification of nucleosides with boron clusters.

Most traditional molecules deal with less than 10 elements (mainly C, H, N, O, S, P, Cl, Fe), whereas metal and semi-metal-containing compounds allow properties that can be gained through the inclusion of nearly 100 additional elements. The variety of molecules containing metal and metal-like elements is extremely large not only because of the larger number of metallic and metalloid elements, but also because of the diversity of available oxidation states, the use of combinations of different metals and the ability to include a plethora of organic moieties organized in the form of low molecular compounds or macromolecular structures [38]. One of the most useful platforms for such new materials are nucleic acids. Nucleic acids constructs used for technological applications consist of two clearly different segments, one is the nucleic acid itself playing a role of information bearing platform, and the second – a function providing modification, in this case a metal or metal carrying complex [14].

Recently we described several methods allowing for incorporation of boron clusters and their complexes with metals (metallacarboranes) into nucleosides, nucleotides [13,14] and nucleic acids [17], some of them are shown in this chapter. Herein we propose a new, and versatile approach based on Huisgen type cycloaddition reaction [39] for modification as well pyrimidine as purine nucleosides.

The Cu(I)-catalyzed 1,3-dipolar cycloaddition of azide and alkyne to form a triazole, termed "click chemistry", has been recently established as an important tool for chemical and biological modification of biomolecules [40]. The 1,2,3-triazole functions as rigid linking unit that can mimic the atom placement and electronic properties of a peptide bond without the same susceptibility to hydrolytic cleavage. The reactants, alkyne and azide, are convenient to introduce, independently, stable, and do not react with common organic reagents or functional groups in biomolecules (are orthogonal). All this factors allow envisioning application of some "click chemistry" approaches not only in general organic synthesis or its offshoots such as synthesis on solid supports or combinatorial chemistry but also in emerging field of "organic chemistry *in vivo*" [41].



Scheme 4. i. Azide/$CuSO_4 \cdot 5H_2O$/potassium ascorbate, *tert*-butanol/water (1:1).

The target nucleoside-boron cluster conjugates were obtained in a simple, one-step procedure (Scheme 4). The reaction was performed under standard "click chemistry" version of Huisgen azide-alkine cycloaddition [42]. Suitable nucleoside acceptor with a spacer of different type and length terminated with ethyne or azide group (not shown) was dissolved in a mixture of *tert*-butanol and water, together with equimolar amount of suitable boron cluster donor equipped with a 3-oxa-pentoxy- spacer terminated with azido or 2-propyn-1-oxy- or 4-pentyn-1-oxy- substituent (not shown). To the obtained solution a catalytic amount of $CuSO_4$ and potassium ascorbate solutions were added. Reactions were performed at room temperature during 8-50 h (usually 24 h) with a TLC control. After reaction completion the solvents were evaporated and the crude product was purified by silica gel column chromatography. The yield of purified product ranged usually from 30 to 65% [43,44].

Proposed approach provides a convenient methodology for synthesis of libraries of boron cluster modified nucleosides for various applications.

Use of nucleoside/boron cluster conjugates range from boron rich boron carriers for BNCT, antiviral agents, modulators of some receptors' activity, modified domains of therapeutic nucleic acids, IR label and others. The applications of boron clusters as versatile electrochemical label will be discussed in more details in one of the following chapters.

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BEHAVIOR OF METALLACARBORANES IN AQUEOUS SOLUTIONS AND THEIR INTERACTION WITH SURFACTANTS AND POLYMERS

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Summary

Polyhedral carboranes, e.g., [3-cobalt(III)bis(1,2-dicarbollide)]$^-$ anion, are known for their hydrophobicity, or at least amphiphilicity. This attribute manifests itself in a surface activity, even when they lack an amphiphilic topology. It leads to formation of small subunits consisting of several metallacarborane molecules bind together by hydrophobic interaction and hydrogen bonding in water. Counterions of the charged metallacarborane clusters play an important role in a stabilizing of the aggregates in aqueous solution. As a result, nanoparticles with typical radius of ca. 100 nm are formed in aqueous solutions depending on aging, concentration and ionic strength. Boron clusters can change dramatically an aggregation behavior of their conjugates with "very water soluble" species (like nucleosides or porphyrines). The light scattering measurements showed that the aggregated boron clusters can interact with conventional surfactants. Due to the formation of hydrogen and dihydrogen bonds, carboranes can interact also with polymer chains. For example, bis(dicarbollide) cobaltate anion decreases solubility of poly(ethylene oxide) and poly(2-vinylpyridine) in aqueous solutions. The phase–separation is induced by presence of inorganic cations in case of poly(ethylene oxide).

Key words: Polyhedral boron clusters, aggregation, surfactants, polymer complexes.

Introduction

A large group of pharmacologically active compounds could be characterized by a common attribute – either their hydrophobic or amhiphilic nature. As a result, these compounds tend to form different assemblies (aggregates) in aqueous solutions [1,2] which could influence significantly their biological activity. The incorporation of a drug into an aggregate affects its physicochemical properties, pharmacokinetics and interaction with biological membranes [3].

A strong hydrophobicity, or at least amphiphicity, is an inherent property of most boron clusters used in the biological and pharmaceutical targeted studies. It has been demonstrated that carboranes enhance hydrophobic interactions between the boron-coupled pharmaceuticals and their receptors, and increase the in vivo stability of compounds that would be otherwise rapidly metabolized [4-7]. The carborane modification also facilitates the cellular uptake due to an increased lipophilicity and easier penetration of the carborane-bearing compounds through cellular membranes [8].

Thus the tendency of metallacarboranes to associate in aqueous solutions could be easily expected, even when they lack an amphiphilic topology. It is obvious that the aggregation process could reduce the concentration of active boron-containing molecules in the solution. Because the incorporation of any drug into an aggregate strongly affects its pharmacokinetics and its biological activity in general, further studies of aggregation of boron-cluster-containing molecules are needed [9,10].

We have focused our research mainly on the formation and behavior of metallacarborane colloidal particles in aqueous solution of a metallacarborane inhibitor of HIV protease, bis(1,2-dicarbollide)cobaltate(l–) (see, Figure 1). We have been studying also other metallacarborane structures like bis[(3)-1,2-dicarbollylcobalt]-(3,6)-1,2-dicarbacanastide-(2–) ions (see, Figure 1) as well as several types of boron cluster conjugates with exoskeletal substitution [11-13].

The fact that some boron-containing structures form aggregates in solution can deteriorate their properties in potential applications. It is well known that hydrophobic compounds can disperse in the surfactant micelles, or at least interact with single amphiphilic molecules [12]. From this point of view, an extensive study on interactions of the boron clusters with surfactant micelles is needed. Besides the low-molecular-mass surfactants, another group of amphiphilic systems is represented by block copolymer micelles which offer unique properties. Furthermore, almost no studies have been done on a solution behavior of carborane with polymers in water. In this communication, we summarize our already published and recently obtained results on the aggregation of boron clusters in aqueous solutions.

Experimental section

Chemicals and Solutions

Cesium salts of metallacarboranes were a kind gift of Dr. Bohumír Grüner and Dr. Jaromír Plešek (Institute of Inorganic Chemistry, Academy of Science of the Czech Republic, Řež near Prague). Structure and preparation of boron cluster conjugates with nucleoside are given elsewhere [12]. Triton X-100 was purchased from Merck KGaA (Darmstadt, Germany). The poly(2-vinylpyridine), PVP, and poly(ethylene oxide), PEO, liner polymers were purchased from Sigma-Aldrich and Fluka, respectively. They are characterized as follows: molar masses of PVP were 15600, 37500, 121500 and 159000; molar masses of PEO were 25800 and 41500. The polydispersity of all samples was in range 1.05-1.15.

Techniques

Dynamic Light Scattering *(DLS).* The light scattering setup (ALV, Langen, Germany) consisted of a 633 nm He-Ne laser, an ALV CGS/8F goniometer, an ALV High QE APD detector and an ALV 5000/EPP multibit, multitau autocorrelator. The data analysis was performed by fitting the measured normalized autocorrelation function $g_2(t) = 1 + \beta|g_1(t)|^2$, where $g_1(t)$ is the electrical field correlation function, t is the lag-time and β is a factor accounting for deviation from the ideal correlation. An inverse Laplace transform of $g_1(t)$ with the aid of a constrained regularization algorithm (CONTIN) provides the distribution of relaxation times, $\tau A(\tau)$

$$g_1(t) = \int_{-\infty}^{\infty} \tau A(\tau) \exp(-t/\tau) \mathrm{d}\ln\tau \qquad (1)$$

Diffusion coefficients were calculated from individual diffusion modes as $D = \Gamma/q^2$, where $\Gamma = 1/\tau$ and $q = (4\pi n_0/\lambda)\sin(\theta/2)$ is the magnitude of the scattering vector. Here θ is the scattering angle, n_0 the refractive index of pure solvent and λ the wavelength of the incident light. Effective angle- and concentration-dependent hydrodynamic radii, $R_H(q,c)$, were obtained from the mean values of relaxation times, $\tau_m(q,c)$, of individual diffusive modes using the equation

$$R_H(q,c) = \frac{kT\tau_m(q,c)q^2}{6\pi\eta_0} \qquad (2)$$

where k is the Boltzmann constant, T is the temperature and η_0 is the solvent viscosity. To obtain true hydrodynamic radii, the data have to be extrapolated to a zero scattering angle. To obtain information on polydispersity, the analysis of the cumulant expansion of the correlation function is performed by fitting a third order polynomial to the function $\ln(g_2(t)\text{-}1)$. The polynomial coefficients

are converted into the coefficients of the cumulant expansion of the field correlation function $g_1(t)$. The Polydispersity Index, PDI, is calculated from the second moment of third order fitting. The PDI can be racalculated in Polydispersity (PD) by simple addition of unity.

Static Light Scattering (SLS). The measurements were performed on the same instrument, which was employed for DLS measurements. Data were treated by the standard method using Zimm equation

$$\frac{4\pi^2 n_0^2 (\mathrm{d}n/\mathrm{d}c)}{\lambda^4 N_\mathrm{A}} \frac{c}{R^{\mathrm{cor}}(q,c)} = \frac{1}{M_\mathrm{w} P(q)} + 2A_2 c \qquad (3)$$

where n_0 is the refractive index of the solvent, dn/dc the refractive index increment of the colloidal particles with respect to the solvent, λ the wavelength of the incident light, N_A the Avogadro constant, $R^{\mathrm{cor}}(q,c)$ is the corrected Rayleigh ratio, which depends on the colloid concentration c and on the magnitude of the scattering vector, $q = (4\pi n_0/\lambda)\sin(\vartheta/2)$, where ϑ is scattering angle, M_w is the apparent weight-average molar mass of scattering particles, A_2 is the "light-scattering-weighted" second virial coefficient of the concentration expansion, and $P(q) = (1+R_\mathrm{g}^2 q^2/3+ \ldots)^{-1}$ is the particle scattering function (R_g is the radius of gyration). Since the values of refractive index increment (dn/dc) and actual concentration of scattering particles are unknown in our systems, SLS data was used only to obtain form factor $P(q)$ by dividing extrapolated values of $Kc/R(c)$ to the zero angle by apparent values of $Kc/R(q,c)$, where c was concentration of carboranes in solution and (dn/dc) value was arbitrary. All solutions of cobalt containing metallacarboranes, measured by light scattering, were colored however a wavelength of used laser (633 nm) does not coincide with absorption bands of metallacarboranes in water. Therefore all SLS and DLS results are reliable.

Small Angle X-ray scattering (SAXS). Measurements were performed on an upgraded Kratky camera with a 60 μm entrance and a 42 cm sample-to-detector distance. Ni-filtered Cu Kα radiation (λ = 0.154 nm) was recorded with a linear position-sensitive detector. The experimental SAXS curves are presented as a function of the magnitude of the scattering vector $q = (4\pi n_0/\lambda)\sin(\vartheta/2)$, where ϑ is scattering angle. Molecular weight M_r can be determined from the forward scatter $I(0)$ (scattering intensity at $\vartheta = 0$) as follows:

$$I(0) = \kappa M_\mathrm{r} c N_\mathrm{A} \left(\frac{n_\mathrm{e}}{M_\mathrm{r}} - \frac{\nu \rho_\mathrm{s}}{N_A} \right)^2 \qquad (4)$$

where κ is an instrumental parameter, c is the weight concentration, N_A is Avogadro's number, n_e is the number of electrons, ν is the partial specific volume of the solute and ρ_s is the mean electron density of the solvent.

Atomic Force Microscopy (AFM). All measurements were performed in the tapping mode under ambient conditions using a commercial scanning probe microscope, Digital Instruments NanoScope dimensions 3, equipped with a Nanosensors silicon cantilever, typical spring constant 40 N m^{-1}. Metallacarborane particles were deposited on a fresh (i.e., freshly peeled out) mica surface (flogopite, theoretical formula $KMg_3AlSi_3O_{10}(OH)_2$, Geological Collection of Charles University in Prague, Czech Republic) by a fast dip coating in a aqueous solution of sample. After the evaporation of water, the samples for AFM were dried in vacuum oven at ambient temperature for ca. 5 hours.

Aggregation of cobaltacarboranes in water [11].

[3-Cobalt(III)bis(1,2-dicarbollide)]$^-$ anion, **1**, exhibits surface activity [14-16]. Therefore, aggregation tendency can be easily expected. The scattering experiments are the best way how we can reveal even low fraction of the aggregates in solution. We used two scattering techniques: the light scattering (LS) and the small angle X-ray scattering (SAXS). Each method is sensitive to different size scatterers.

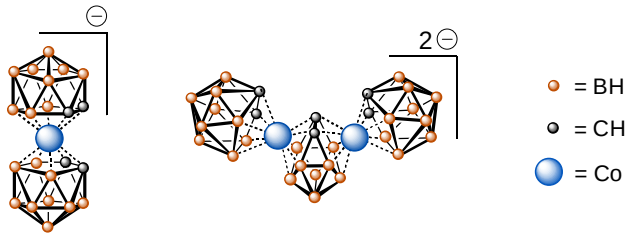

Figure 1. Structures of polyhedral metallacarboranes with peanut- like (**1**) and banana-like (**2**) shapes.

Scattering at visible light wavelengths is dominated by nanoparticles with a diameter of several tens or hundreds of nanometers. Due to the value of q^{-1} of X-ray, the SAXS measurements can distinguish even much smaller objects (from several Angstroms to several nanometers). The methods differ also in a contrast. The LS intensity is high when the solution has a large value of the refractive index increment. In case of SAXS, the contrast is defined as the difference in the mean electron densities of the solute and solvent and it is proportional to the partial specific volume of the studied particles (for **1**, it was determined to be 0.7 mL/g).

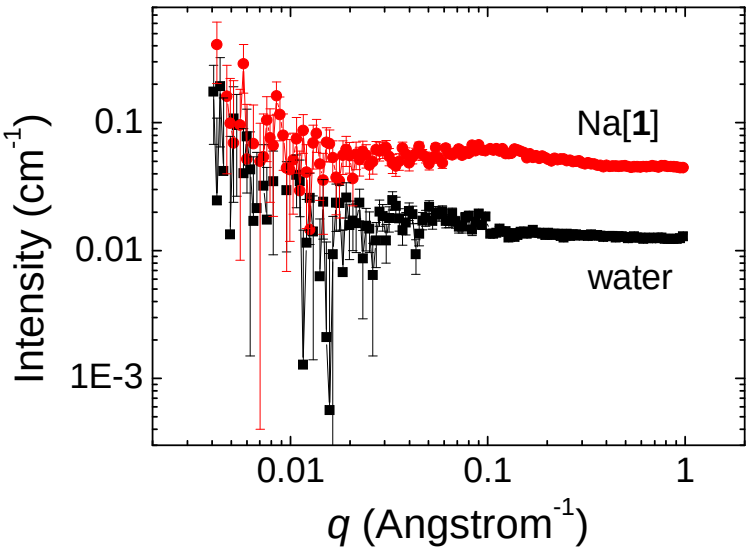

Figure 2. The SAXS curves of pure water and Na[**1**] aqueous solution with concentration 50 g/L.

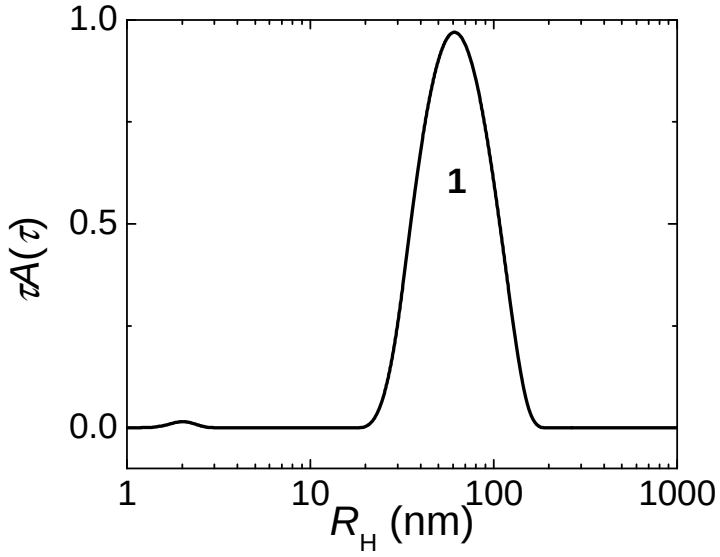

Figure 3. Typical DLS distribution of hydrodynamic radii, R_H, of Na[**1**] aggregates with concentration 0.3 g/L two days after solution preparation.

Typical X-ray scattering curve is shown in Figure 2. The sodium cobalt bis(dicarbollide) concentration has to be very high to obtain reliable data. From the corresponding "featureless" curve, it is evident that there are no compact particles larger than $\lambda/20$ (ca. 3nm) and lower than the SAXS limit in the solution. However, from the increase of the intensity comparing to the solvent we can calculate whether the cobaltacarborane is molecularly soluble at given concentration (using Equation 4). The result is quite surprising. The **1** anion forms small clusters of in average 5 molecules. Similar result was obtained for lower concentrations (to 8 g/L), but with a higher degree of uncertainty.

The LS results are much more complex than the SAXS ones. From both SLS and DLS measurements, we can unambiguously conclude that all studied aqueous solutions of **1** and **2** anions (schemes in Figure 1) contain large nanoparticles. The solutions are stable and do not precipitate. Typical distribution of hydrodynamic radii, R_H, of Na(**1**) aggregates is plotted in Figure 3. The distribution is monomodal and with relatively low index of polydispersity. It is worth-mentioning that the DLS distributions are always monomodal, but the position and width of the peak depend significantly on aging and solute concentration. The freshly prepared solutions of Na(**1**) with concentration larger than 0.25 g/L are very polydisperse even after a careful filtration through a 0.2 μm membrane filter. After several days, the nanoparticles become relatively monodisperse with R_H around 110 nm. Very dilute salt free solutions of **1** behave very peculiar. The distributions are very narrow and R_H increases steeply with decreasing concentration (up to 300 nm). This microphase separation can be sufficiently suppressed by addition of indifferent salt. The Cs(**1**) is less soluble than the sodium salt, but the effects are comparable. Double charged anion **2** does not behave so complex, although it forms also nanoparticles with radius of ca. 110 nm.

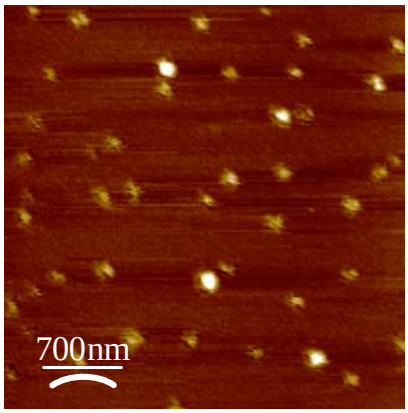

Figure 4. Typical AFM scan of Na[**1**] aggregates deposited on a dry mica surface.

From the SLS measurements, we can obtain the radius of gyration, R_g, of the nanoparticles. We divided these values by corresponding R_H and compared it with theoretical R_g/R_H values for monodisperse spheres (0.775). The ratios for **1** and **2** aggregates are very close to this prediction. Together with the AFM scans of the cobaltacarborane aggregates on mica as shown in Figure 4, we can assume from the value of the ratio that the nanoparticles have roughly spherical shape. Nevertheless, we should keep in mind that the R_g/R_H ratio can be obscured by polydispersity and that the particles are deposited on a dried mica surface.

Seemingly, there is a contradiction between the SAXS and the LS results. Fortunately, we can explain the presence of the particles with both sub-nanometer and hundred nanometer sizes in the system as follows. (I) The very small aggregates do not sufficiently scatter the visible light. (II) The large nanoparticles are out of the SAXS scale range. (III) The fraction of the large aggregates can be quite high but the nanoparticles consist of subunits of five molecules. Another explanation that SAXS did not detect the nanoparticles due to the very low fraction of the nanoparticle, is not likely correct. To obtain information on the fraction of aggregated cobaltacarborane molecules, a gel permeation chromatography study of Na[**1**] solutions were carried out. All chromatograms for concentrations between 1 and 10 g/L are fully reproducible and show two separated peaks (not shown). The first peak can be assigned to aggregates and the second one to the fraction of individual molecules or small oligomers, which are detectable neither by LS nor by AFM. From the area of both peaks we determined the fraction of small oligomers and large nanoparticles to be ca. 20% and 80%, respectively.

Aggregation of nucleoside–boron cluster conjugates in water [12].

In this section, we report our observations concerning the behavior of nucleosides substituted by boron clusters in aqueous media. The combined LS and AFM study revealed an intricate balance between hydrophobic and hydrophilic interactions, further, a sensitivity of the behavior to the nature of boron clusters. The size, charge, and exoskeletal pattern of the boron cluster can strongly influence the aggregation. The nucleobases with attached electroneutral (dicarba-*closo*-dodecaboranyl) or bulky charged ([3-cobalt(III) bis(1,2-dicarbollide)]$^-$ anion) clusters aggregate spontaneously, whereas the nucleobases containing smaller and charged *nido*-clusters (dicarba-*nido*-undecaboranyl) as well as the unmodified nucleosides remain in the form of true solutions or oligomolecular associates that are not observable by LS. The aggregation is strongly enhanced by the presence of amino groups, because of the formation of zwitterions which are sparingly soluble in the aqueous solution [17-18].

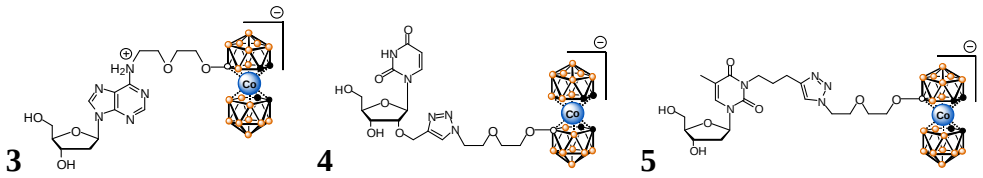

Figure 5. Chemical structures of -*N*-{5-[3-cobalt(III) bis(1,2-dicarbollide)-8-yl]-3-oxa-pentoxy}-2'-*O*-deoxyadenosine – **3**, 2'-*O*-methyl-(1,2,3-triazol-4-yl)-{4-*N*-{5-[3-cobalt(III) bis(1,2-dicarbollide)-8-yl]-3-oxa-pentoxy}}-uridine – **4** and 3-*N*-propyl-(1,2,3-triazol-4-yl)-{4-*N*-{5-[3-cobalt(III) bis(1,2-dicarbollide)-8-yl]-3-oxa-pentoxy}}-thymidine – **5**.

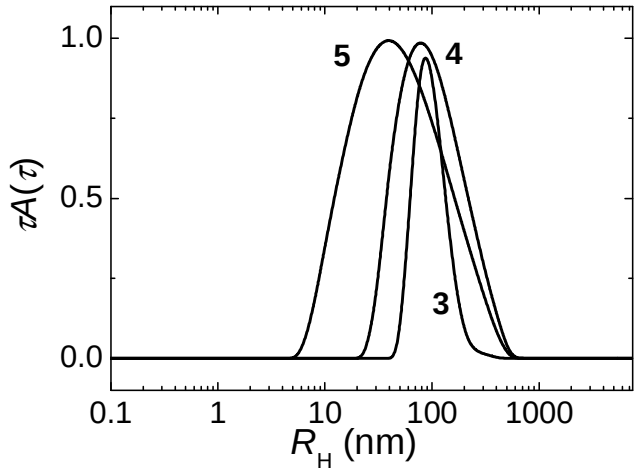

Figure 6. Typical DLS distribution of hydrodynamic radii, R_H, of **3**, **4** and **5** aggregates. The concentrations were 1.78×10^{-5} M for **3**, and 1.00×10^{-4} M for **4** and **5**.

Here, we will discuss the representative results of nucleoside conjugates with cobalt bis(dicarbollide) anion only (chemical structures depicted in Figure 5). As compared with the parent boron cluster (**1**), the conjugates form nanoparticles in a certain concentration range only with radii around 100 nm. Typical R_H distributions are shown in Figure 6. The presence of highly hydrophilic and therefore very water soluble nucleoside part introduces an amphiphilic topology into **3** - **5** molecules. Therefore, aggregation behavior resembles that of conventional surfactants. It means that the aggregates can disappear below a certain concentration – the critical association concentration (cac), which was not detected for the parent cluster. (**11**)

Assuming that the cac of **4** and **5** aggregates is ca. 1×10^{-4} M, the behavior of the compounds seems to obey the mechanism of the closed association (this is typical for surfactants and polymeric micelles). Nevertheless, a fraction of the aggregated **4** and **5** molecules above the cac is much lower than in the case of **1**. In the case of solutions **3**, the situation is different due to the presence of amino groups and formation of sparingly soluble zwitterions [17-18]. The system containing compound **3** forms only an insignificant number of the nanoparticles indicating that the pre-aggregation takes place below the concentration of 5×10^{-6} M. Above this limit the fraction of the aggregates increases considerably. This concentration can be considered to be the apparent cac, although the aggregation number as well as the concentration of non-aggregated molecules seems to change above the cac.

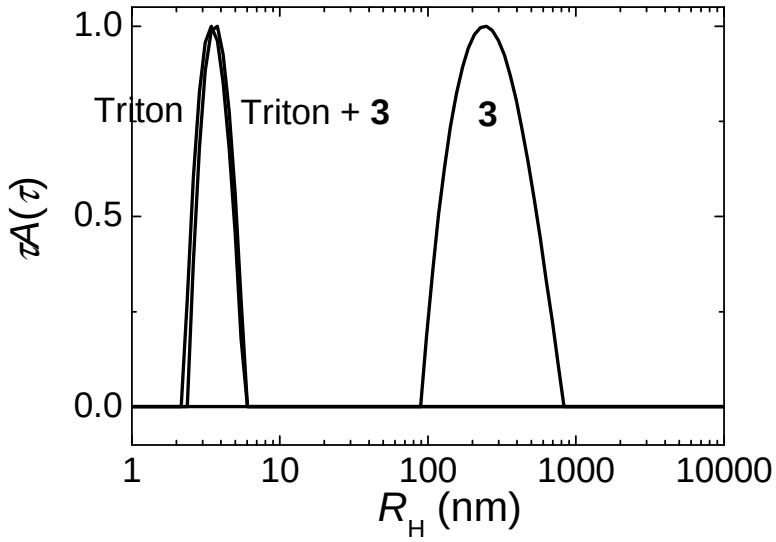

Figure 7. DLS distribution of hydrodynamic radii, R_H, of Triton X-100, **3** aggregates and their mixture in water. The concentrations were 1.00×10^{-4} M for **3**, and 0.25% for Triton X-100.

Interaction of boron clusters with non-ionic surfactants [12].

The light scattering measurements also showed that the aggregated species can interact with nonionic micelles in the solution. We carried out light scattering experiments with nonionic surfactant Triton X-100 which forms micelles with the radius of ca. 3 nm. It is well known that hydrophobic compounds can disperse in the surfactant micelles. The solutions above the cac of compounds **3** - **5** were studied in a presence of Triton X-100 with concentration of 0.25% by LS. This is well above the cmc of Triton (ca. 0.13

%). The compound **3** dispersed in the surfactant micelles quantitatively as indicated by significant decrease of the LS intensity and diminishing of the **3** peak in the R_H distribution (see Figure 7). Within the uncertainty due to low LS intensities, the same is true also for the species **4** and **5**.

Interactions of the studied compounds with the Triton X-100 molecules were also observed in the solutions below the cmc of Triton (0.01 %). The radii of large nanoparticles of **3** decreased to ca. 105 nm, though the fractions of the aggregated molecules remain quite high. All these observations are not so surprising when assuming that the considered boron conjugates exhibit amphiphilic and surface active behavior like surfactants.

Interaction of cobaltacarboranes with linear polymers

In respect to possible pharmacological applications for drug-delivery systems, [19] we studied interactions of **1** with several high molecular weight polymers. (I) Polyanions like poly(acrylic acid) and poly(methacrylic acid) do not seemingly interact with **1** and were not studied in detail. (II) Polycations containing a protonizable nitrogen group - poly(2-vinylpyridine), PVP, form the sparingly soluble zwitterionic complex with **1** as expected. (III) Hydrophilic non-polyelectrolyte as poly(ethylene oxide), PEO, the behavior of which deserves our attention. We observed that even small amount of **1** can lead to the phase separation or at least aggregation. The precipitate contains both PEO chains and cobaltacarborane molecules, where the ratio of **1** to overall number of PEO segments in the system depends strongly on the cation concentration in the bulk solution. In the other words, the **1** uptake by PEO chains increases significantly with the concentration of, e.g., sodium chloride. It is also worth-mentioning that the uptake/release lasts several days or weeks. The boron cluster/polymer complex formation can be explained by formation of classical hydrogen and peculiar dihydrogen bonds [20,21].

From analysis of the **1**/polymer mixtures near equilibrium, we can calculate that the PVP-based complex contains in average 2.5 polymer segments per one **1** molecule, while the PEO-based complex contains at least ca. 10 segments per **1** depending strongly on the sodium ions concentration. However, the studied structures likely have not a stoichiometric composition. Above described phenomenon manifests itself in the pronounced aggregation or micellization of copolymers which contain PVP and/or PEO blocks in their architecture.

Acknowledgment

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BORONIC ACID AS AN ALTERNATIVE FUNCTIONAL GROUP FOR DRUG DESIGN

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Summary

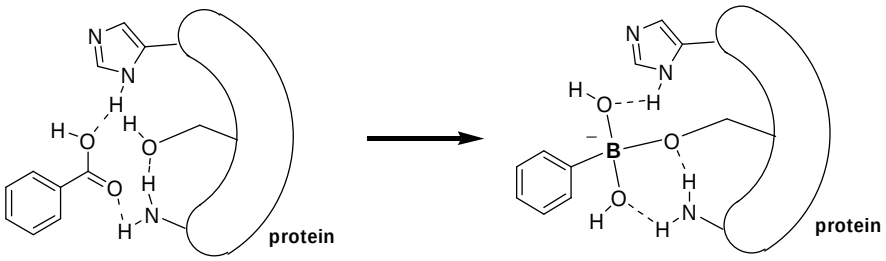
Aminoboronic acids as growth-factor receptor tyrosine kinase inhibitors and boronic acid containing *cis*-stilbenes as tubulin polymerization inhibitors were designed and synthesized based on the biologically active structures of Lavendustin **1** and Combretastatin A-4, respectively. The selective inhibitory activities were observed in a series of the aminoboronic acids: 4-Methoxy-3-[(2-methoxy-phenylamino)-methyl]-phenylboronic acid **9** inhibited EGFR tyrosine kinase, whereas 4-(2,5-dihydroxy-benzylamino)-phenylboronic acid **10** inhibited Flt-1 protein kinase, although lavendustin pharmacophore **1** inhibited both EGFR and Flt-1 kinases at a 1.0 μg/ml concentration of the compound. High cell growth inhibitions were observed in boronic acid containing *cis*-stilbenes **20c** and **20d**, in which a hydroxyl group on the aromatic ring B of the combretastatin A-4 was replaced by a boronic acid, and their IC_{50} values toward B-16 and 1-87 cell lines are 0.48-2.1 μM. The compounds **20c** and **20d** exhibited a significant inhibitory activity of tubulin polymerization (IC_{50} = 21-22 μM). Growth Inhibitory experiments against a Panel of 39 human cancer cell lines revealed that **20c** showed differential growth inhibition in comparison with Combretastatin A-4 and their correlation coefficient (r) was 0.553 in the COMPARE analysis.

Key words: boronic acid, covalent bond interaction, tyrosine kinase inhibitor, *cis*-stilbenes, tubulin polymerization inhibitor, COMPARE analysis

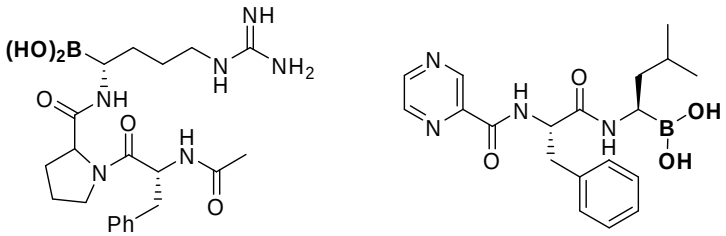
Introduction

The utility of boron atoms as a material for pharmaceutical drug design possesses a high potential for a discovery of new biological activity [1]. A boron atom has a vacant orbital and interconverts with ease between the neutral sp^2 and the anionic sp^3 hybridization states, which generates a new stable interaction between a boron atom and a donor molecule through a covalent bond [2,3]. Therefore, it is expected that the boron atoms introduced into biologically active molecular frameworks would interact with a target protein not only through hydrogen bonds but also through covalent bonds, and this interaction would produce a potent biological activity (Scheme 1) [4]. Among

various boron compounds synthesized, recently much attention has been paid for a boronic acid containing peptides [5]. Fevig and the co-workers have developed a boropeptide as a thrombin inhibitor [6,7]. In those boron peptides, a carboxylic acid was replaced by a boronic acid. Bortezomib (originally PS-341 and marketed as Velcade) is a dipeptidyl boronic acid and the first therapeutic proteasome inhibitor to be tested in humans [3,8]. It was approved by the FDA for treating relapsed multiple myeloma and mantle cell lymphoma in 2003. In multiple myeloma, complete clinical responses have been obtained in patients with otherwise refractory or rapidly advancing disease.



Scheme 1. Interaction of a carboxylic acid through hydrogen bonds (left) and interaction of a boronic acid through both hydrogen and covalent bonds (right) to a certain protein.



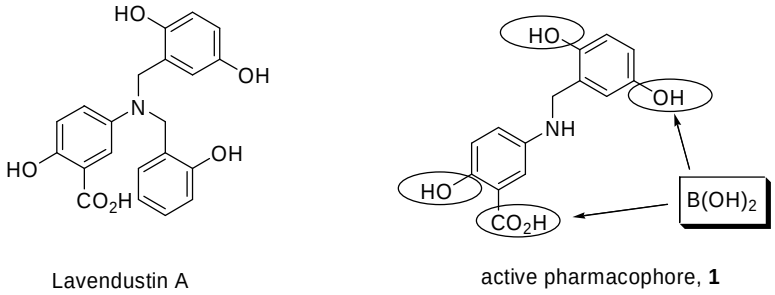
Scheme 2. Structures of boron peptides as enzyme inhibitors.

According to the X-ray structural analysis, the boronic acid moiety interacts with Thr-1 of chymotryptic-like active site of proteasome to form sp3 boron though covalent bonding [3]. This covalent interaction cause the reversible inhibitory activity of Bortezomib.

Our strategy for the design of boron compounds is based upon their unique properties that make them different from conventional biologically active compounds. In this paper, design, synthesis, and biological property of aminoboronic acids as growth-factor receptor tyrosine kinase inhibitors [9] and boronic acid containing *cis*-stilbenes as tubulin polymerization inhibitors [10] are described.

Aminoboronic acids as Growth-Factor Receptor Inhibitors

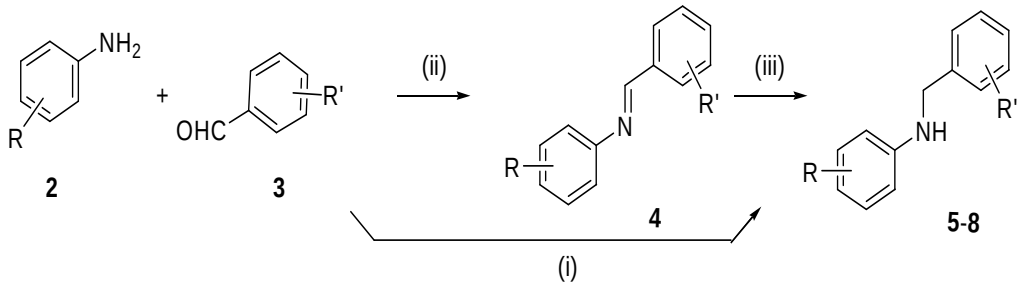
Lavendustin A is the epidermal growth-factor receptor (EGFR) protein tyrosine kinase (PTK) inhibitor isolated from a butyl acetate extraction of *Streptomyces griseolavendus* culture filtrate [11]. The active pharmacophore **1** is a secondary amine containing three phenol-type hydroxy groups and a carboxylic acid, and considered to interact with EGFR-PTK through hydrogen bonds at those functional groups (Scheme 3) [12]. We focused on the active pharmacophore **1** of Lavendustin A and introduced a boronic acid into this pharmacophore.



Lavendustin A active pharmacophore, **1**

Scheme 3. Introduction of boronic acid into the active pharmacophore of lavendustin A.

The active pharmacophore **1** of lavendustins composed of two aromatic rings linked by a methyleneamino chain and it was previously synthesized from 3-aminosalicylic acid and 2,5-dihydroxybenzaldehyde by reductive amination reaction.

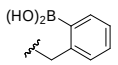
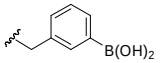
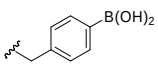
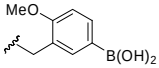
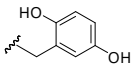
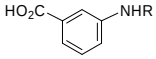
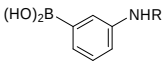
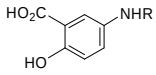


Scheme 4. General synthesis of aminoboronic acids. Reagents and conditions: (i) $NaCNBH_3$, MeOH, room temperature, (ii) MeOH or EtOH, (iii) $NaBH_4$, EtOH.

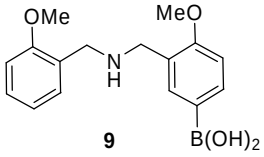
A series of aminoboronic acid analogues **5-8** was synthesized from various anilines **2a-d** and aldehydes **3a-e** in the presence of $NaCNBH_3$ in MeOH at room temperature or $NaBH_4$ in EtOH after the formation of the imines **4**

(Scheme 4). Structures and yields of the aminoboronic acids **5-8** are summarized in Table 1.

Table 1. Structures and Yields of the Aminoboronic Acids **5-8**.

aniline	R = 				
	**5a**: 86%	**5b**: 81%	**5c**: 39%	**5d**: 16%	—[a]
	**6a**: 20%	**6b**: 31%	**6c**: 74%	**6d**: 15%	**6e**: 73%[b]
	**7a**: 58%	**7b**: 28%	**7c**: 39%	**7d**: 23%	**7e**: 90%
	—[a]	**8b**: >99%	**8c**: >99%	**8d**: 47%	**1**: 50%[b]

[a] Not synthesized. [b] Already reported in ref 22.



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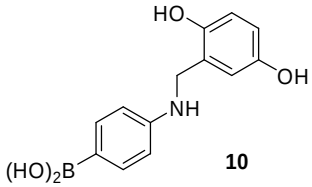


10

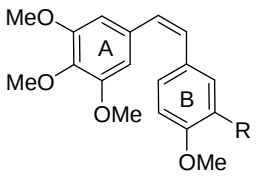
Selective Inhibition Assay of Various Tyrosine Kinase Activity.

The lavendustin pharmacophore **1** and the aminoboronic acids **5a-d**, **6a-d**, **7a-e**, **8b-d**, **9**, and **10** were tested for the inhibitory activity of EGF-stimulated EGFR tyrosine kinase phosphorylation according to assay conditions described in detail in the literature [13]. Inhibition specificity of the compounds was investigated semi-quantitatively using PKA, PKC, PTK, eEF2K, EGFR, and Flt-1. The lavendustin pharmacophore **1**, the aminoboronic acid **6b**, and the analogue **6e** showed selective kinase inhibitions of both EGFR and Flt-1. The selective inhibition of EGFR tyrosine kinase was observed in the case of the aminoboronic acids **6c**, **8c**, and **9**. It is considered that the substitution of a boronic acid at the *para* position instead of the dihydroxy groups of the benzyl moiety in **1** enhances the specific inhibition of EGFR tyrosine kinase. Although the compound **7e** did not show the inhibition of EGFR kinase, the specific inhibition activity was observed in the case of Flt-1 kinase assay at a concentration of 10 μg/ml. The compound **10** exhibited

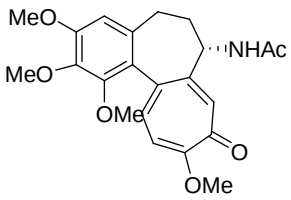
nonspecific inhibition activity toward various protein kinases at a concentration of 10 μg/ml but the specific inhibition activity of Flt-1 kinase was observed at 1.0 μg/ml. Since the compounds, **6e**, **7e**, and **10** contain a 2,5-dihydroxybenzyl group, it is considered that the selective inhibition of Flt-1 by **7e** and **10** is due to a boronic acid group on the aniline ring in the molecules, and the *para*-substituted boronic acid increased the inhibitory activity.

Boronic Acid Containing *cis*-Stilbenes as Tubulin Polymerization Inhibitors.

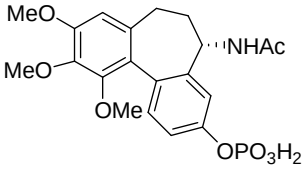
Combretastatin A-4 isolated from the bark of the South African tree *Combretum caffrum* by Pettit and the co-workers [14] has been found to be a potent inhibitor of the tubulin polymerization as well as a cytotoxic agent against a wide variety of human cancer cell lines including multidrug resistant cancer cell lines, therefore it is an attractive lead compounds for development of anticancer drugs [15]. CA-4P and ZD6126 are water-soluble sodium phosphate prodrugs of combretastatin A-4 and allocolchinol, respectively, and currently in phase I/II clinical trials [16]. AC-7739 contains the HCl salt of the amine instead of the C-3 hydroxyl group in the aromatic ring B of combretastatin A-4 and AC-7700 (AVE-8062) is the L-serine amide HCl salt. Combretastatins and these analogues have a donor-type functional group, as a common structure, substituted on the aromatic ring B. We have been interested in the introduction of *a boronic acid as an acceptor-type functional group* into the aromatic ring B in the combretastatin framework.



11a: R = OH, Combretastatin A-4
b: R = OPO_3Na_2, CA-4P
c: R = $NH_3^+Cl^-$, AC-7739
d: R = NH-Ser HCl salt, AC-7700



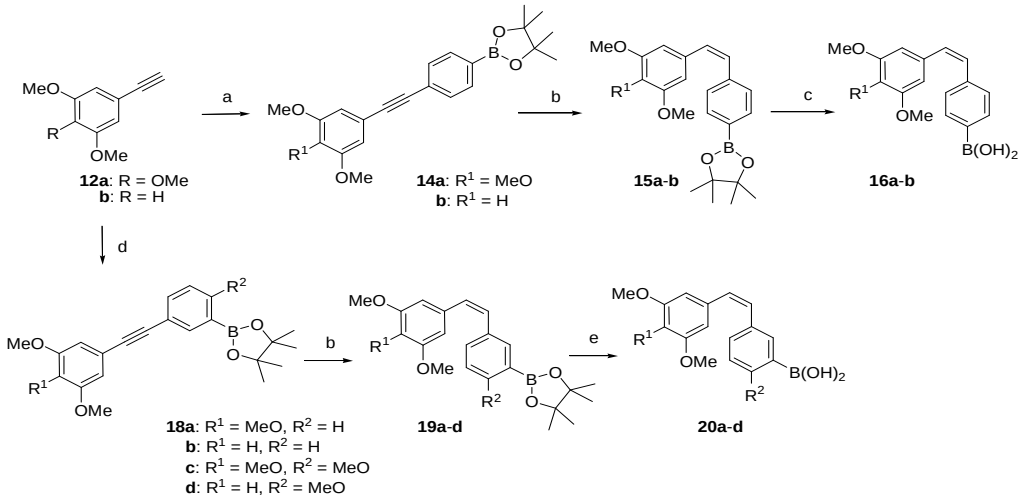
Colchicine



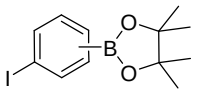
ZD6126

We desinged the synthetic scheme of a boronic acid containing *cis*-stilbene analogues from phenylacetylenes and iodophenylboronic acids through Sonogashira coupling reaction followed by *cis*-reduction [17]. The phenylacetylenes **12a**-**b** were synthesized from the corresponding aldehydes through Corey-Fuchs reaction and Sonogashira coupling reactions between the acetylenes **12a**-**b** and the iodide **13a** gave the corresponding alkyne **14a** in 70 % yield (Scheme 5). *cis*-Reduction of the alkyne **14a** was accomplished by using dicyclohexylborane in THF to give the *cis*-stilbene **15a** in 67% yield.

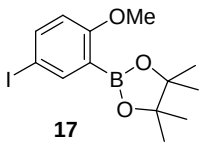
KHF_2 was employed for the deprotection of the ester group on **16a** and the *para*-boronic acid **16a** was obtained in 46 % yield. In a similar manner, the boronic acid **16b** was synthesized from the acetylene **12b** and the iodide **13a** in three steps. The *meta*-boronic acid derivatives **20a-d** were also synthesized from **12a-b**. The acetylenes **12a-b** underwent Sonogashira coupling reaction with the iodides **13b** or **17** to afford the corresponding alkynes **18a-d**. *cis*-Reduction of **18a** with dicyclohexylborane gave the *cis*-stilbene **19a** in 53 % yield and protection of **19a** with KHF_2 gave the desired product **20a** in a very poor yield (8 %). After several trials, we found that the transesterification with diethanolamine followed by hydrolysis under the acidic condition was efficient for the deprotection of the boronic acid pinacol ester **19a** and the corresponding boronic acid **20a** was obtained in 38 % yield by two steps. The boronic acid pinacol ester **19b** underwent the deprotection reaction with diethanolamine to give the boronic acid **20b** in 68% yield. Synthesis of the 4-methoxy-substituted (R^2 = MeO) *cis*-stilbenes **20c-d** was also accomplished. The reaction of the acetylenes **12a-b** with 5-iodo-2-methoxyboronic acid pinacol ester **17** afforded the corresponding alkynes **18c-d**. Use of 5-bromo-2-methoxyboronic acid pinacol ester was not effective for this coupling reaction. Reduction of **18c-d** gave the *cis*-stilbene **19c-d**, which underwent deprotection reaction by the transesterification method to give **20c-d** in 46 % and 68 % yields, respectively.



Scheme 5. Reagents and conditions: (a) **13a**, $Pd(PPh_3)_4$, CuI, TEA, CH_3CN, 70 ºC, 71-73%; (b) dicyclohexylborane, THF, 0 ºC, 40-68%; (c) KHF_2, ether, then HCl, 25-46 %; (d) **13b** or **17**, $Pd(PPh_3)_4$, CuI, TEA, CH_3CN, 70 ºC, 46-82 %; (e) diethanolamine, then HCl, 46-68%.



13a: *p*-$B(OH)_2$
b: *m*-$B(OH)_2$



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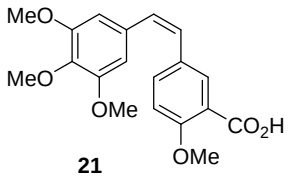

21

The carboxylic acid derivative **21** was also synthesized for comparison with the boronic acid **20c**.

In Vitro Cell Growth Inhibitory Assay.

Cell growth inhibition of boronic acid analogues toward B-16 and 1-87 cell lines was examined. The results are summarized in Table 2. The alkynes **14a** and **14b** exhibited a 50% cell growth inhibition at 20-36 μM concentrations of compounds. Although the *cis*-stilbenes **16a** and **16b**, which have a boronic acid substituted at a para position on the aromatic ring B of a combretastatin framework, exhibited higher cell growth inhibition (2.6-12 μM), the corresponding meta-substituted boronic acids **20a** and **20b** were more effective for cell growth inhibition (0.48-2.1 μM). Finally, we found that the compound **20c**, which has a methoxyl group substituted at a para position on the aromatic ring B, possesses a significant inhibitory activity of cell growth.

Table 2. The Cell Growth and Tubulin Polymerization Inhibitions of Boronic Acid Derivatives

	Cell Line (IC_{50}, μM)[a]		Tubulin Inhibition
compound	B-16[b]	1-87[c]	IC_{50} (μM)
14a	20	22	nd
14b	32	36	nd
16a	2.6	3.2	>100
16b	12	9.1	79
20a	0.49	2.1	>100
20b	1.8	2.0	>100
20c	0.0063	0.013	22
20d	0.019	0.028	21
21	160	110	>100
Combretastatin A-4, **11a**	0.0046	0.0085	1.8
11c	0.0080	0.011	5.0
Colchicine	0.056	0.012	nd

[a] The compounds were assayed at least three times and the IC_{50} values reported here are mean of an average of three experiments. [b] Mouse B-16 melanoma cell line. [c] Human lung carcinoma 1-87 cell line.

The IC_{50} values of **20c** toward B-16 and 1-87 cells are 0.0063 μM and 0.013 μM, respectively, and these inhibitory activities are similar to those of the amide derivative **11c** (B-16 cells: 0.0080 μM, 1-87 cells: 0.011 μM), although Combretastatin A-4 (**11a**) is more efficient for cell growth inhibition (B-16 cells: 0.0046 μM, 1-87 cells: 0.0085 μM). Three methoxyl groups substituted on the aromatic ring A were necessary to obtain a higher cell growth inhibitory activity (**16a, 20a** and **20c** versus **16b**, **20b** and **20d**), as reported by Cushman, Hamel and coworkers [18]. Unprecedentedly, the carboxylic acid derivative **21**, which is considered as a mimic of the boronic acid **20c**, showed 10,000-fold less cell growth inhibition than **20c** toward B-16 and 1-87 cells.

Inhibition of in vitro Tubulin Polymerization.

In order to investigate inhibitory activities of the synthesized boronic acids toward the microtubule system, their effects on in vitro polymerization of tubulin were examined. The tubulin was purified from procine brains according to the Shelanski protocol with modification [19]. The results are also shown in Table 1. Inhibitory activity of the compound **16a** was not observed at a 100 μM concentration, whereas, the compound **16b** exhibited enhanced inhibition and the IC_{50} value was 79 μM. Compounds **20a** and **20b** did not show significant inhibition at a 100 μM concentration of compounds. The methoxy group substituted at a para position on the benzene ring B enhanced their inhibitory effect on tubulin polymerization, and a significant inhibitory activity was observed in the compounds **20c** and **20d** with relatively low IC_{50} values of 22 and 21 μM, respectively. Two methoxy groups substituted at 3 and 5 positions on the ring A were potent for significant inhibition of tubulin polymerization. The carboxylic acid **21**, as a mimic of the boronic acid **20c**, were also tested for the inhibition assay. However, no significant inhibition was observed at a 100 μM concentration. These results indicate that a boronic acid is more effective than a carboxylic acid, although the electron donor groups, such as hydroxy (**11a**) and amine (**11c**), are more suitable for inhibition of tubulin polymerization.

Growth Inhibition against a Panel of 39 Human Cancer Cell Lines.

The compound **20c** and Combretastatin A-4 were tested for in vitro antiproliferative activity against a panel of 39 human cancer cell lines, which is similar to the one developed by the National Cancer Institute [20]. More than 200 standard compounds, including various anticancer drugs and various types of inhibitors, have been evaluated using this pannel. We compared standard drugs with each other for the mean graph pattern using COMPARE analysis and confirmed that drugs sharing a certain mode of action clustered together, as described previously [21]. The patterns in the mean graphs of drugs with common modes of action resembled one another. Figure 1 shows the mean

graphs of the compound **20c** and Combretastatin A-4 based on the growth inhibition parameter of GI_{50}. The compound **20c** showed differential growth inhibition in comparison with Combretastatin A-4. The mean log GI_{50} values of **20c** and Combretastatin A-4 were –7.86 and –8.08, respectively. Enhanced inhibitoty activities of **20c** were observed in cell lines of HT-29, AS49, OVCAR-4, OVCAR-5, and PC-3, and the *Delta* value was 1.14. Less inhibitory activities were observed in those of HBC-4 and MKN45, and the *Range* value was 2.74.

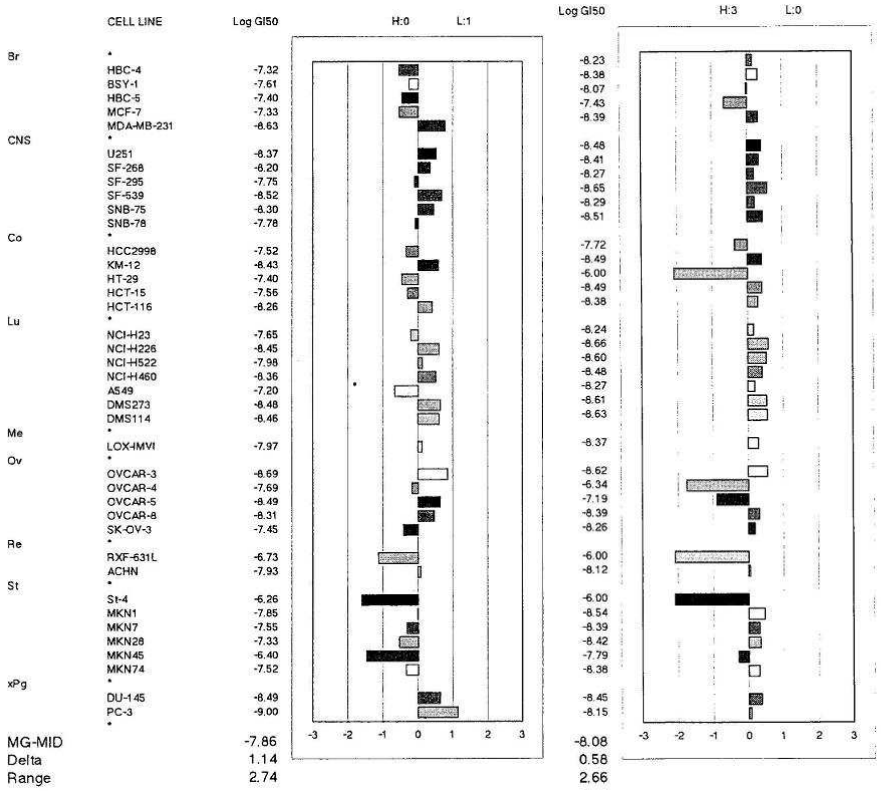

Figure 1. Growth inhibition of **20c** (left graph) and Combretastatin A-4 (**11a**, right graph) against a panel of 39 human cancer cell lines. The mean graph was produced by computer processing of the GI_{50} values as described in the experimental section. The log GI_{50} for each cell line is indicated. *Columns* extending to the *right*, sensitivity to compounds; *columns* extending the *left*, resistance to compounds. One scale represents one logarithm difference. MG-MID, the mean of log GI_{50} values for 39 cell lines.

In the mean graphs of a panel screening, a compound, which has *Delta* and *Range* values of >0.5 and >1, respectively, is evaluated as positive for differential activity, therefore, **20c** and Combretastatin A-4 possess differential activities toward certain cancer cells. The COMPARE analysis of the mean

graphs revealed the correlation ranking with **20c**; Combretastatin A-4 (r = 0.553), Breomycin (r = 0.532) which is a DNA strand break agent, Taxol (r = 0.455), and Vincristine (r = 0.436). In the COMPARE analysis, positive correlation between two agents, which possess similar modes of action, results in r values of higher than 0.75, and a compound, which is correlated to a certain agent with r values of 0.5-0.75, has a possibility of different modes of action in addition to similar modes of action to the agent. Therefore, the current analysis may suggest that the compound **20c** possesses a possibility of different modes of action beside tubulin polymerization inhibition from Combretastatin A-4.

In conclusion, we designed and synthesized a series of boronic acid containing biologically active compounds based on the structures of natural products. Selective inhibition of Flt-1 was observed in **7e** and **10** and it may be due to a boronic acid group on the aniline ring in the molecules. Furthermore, significant tubulin polymerization inhibition as well as cell growth inhibition was observed in the boronic acids **20c-d**. The COMPARE analysis against a anel of 39 human cancer cell lines suggests that **20c** possesses a possibility of different modes of action beside from Combretastatin A-4.

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BORON CLUSTERS AS ELECTROCHEMICAL LABELS FOR BIOMOLECULES

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Summary

Boron clusters and their complexes with metals are proposed as new class of redox labels for biomolecules. A general approach to the synthesis of derivatives of four canonical nucleosides: thymidine (T), 2'-deoxycitidine (dC), 2'-deoxyguanosine (dG), 2'-deoxyadenosine (dA) and their ribo-counterparts bearing metallacarborane complexes with metals such as cobalt, iron, chromium or rhenium is shown. Metallacarborane/nucleoside conjugates are prepared *via* reaction of suitable 8-dioxane-3-metal bis(dicarbollide) adduct with base-activated, protected nucleosides or by *de novo* formation of 3,3,3-tricarbonyl-3-rhenium-1,2-dicarba-*closo*-dodecaborate complex from carborane/nucleoside ligand. The conjugates display easily distinguishable, diagnostic electrochemical signals assigned to boron cluster component. The above finding lays foundation for a "multi color" electrochemical coding of DNA based on electrochemical properties of boron clusters.

Key words: redox labels, boron clusters, nucleoside/boron conjugates, DNA-oligonucleotide probes

Introduction

Molecular diagnostics based on analysis of genomic sequence offers highly sensitive and quantitative methods for detection of infectious disease pathogens and genetic variation. Conventional methods of analysis of gene sequence are based on either direct DNA sequencing or on DNA hybridization technology. Because its simplicity, DNA hybridization approach is more commonly used in the diagnostic laboratories then the direct sequencing method. In DNA hybridization, the target gene sequence is identified by a DNA probe that can form a double-stranded hybrid with complementary nucleic acids with high efficacy and specificity in the presence of a complex mixture of different non-complementary nucleic acids [1].

Wide-scale genetic testing requires the development of easy-to-use, fast, inexpensive, miniaturized analytical devices. Traditional methods for detecting DNA hybridization, such as gel electrophoresis or membrane plots,

are too slow and labor expensive. Radioactive labels are sensitive but have the obvious disadvantage of short shelf life, risk associated with exposure of the personnel to radiation. Biosensors offer a promising alternative for faster, cheaper, and simpler nucleic-acids assays. Such devices intimately couple a biological recognition element with a physical transducer. The major processes involved in any biosensor system are the analyte recognition, signal transduction, and readout. Common transducting elements, include optical, electrochemical, or mass-sensitive devices, generate light, current, or frequency signals, respectively [2].

Electrochemical transducers have received considerable recent attention in connection to the detection of DNA hybridization [3]. Modern electrical DNA hybridization biosensors and bioassays offer remarkable sensitivity, compatibility with available microfabrication technologies, inherent miniaturization, low cost, minimal power requirements, and independence of sample turbidity or optical pathway. Therefore, electrochemical detection of nucleic acids is an attractive alternative to established fluorescence and others optical coding technologies [4]. Various approaches have been explored, including conjugation of DNA oligonucleotides with electroactive reporters, use of soluble electroactive mediators or intercalators, redox enzyme mediation, and measurement of direct label-free electrochemical processes [5,6].

Redox active metal complexes covalently linked to DNA are most frequently used as electrochemical reporters, in particular ferrocene, the metallocene type complex, is the best known example [7]. However, in spite of the fact that many metallocenes with different central metals are available [8], none of them, with the exception of ferrocene, was used till now as DNA-electrochemical label due to air- and/or moisture sensitivity [9]. The only alternative to ferrocene as covalently linked, electrochemically active label for electrochemical detection of nucleic acids described so far are metallacarboranes [10].

Metallacarboranes, discovered by Hawthorne in early sixties are vast family of metallocene type complexes consisting at least one carborane cage and one or more metal atoms. Carborane (borane) clusters are versatile and efficient ligands for metals like Al, Au, Co, Cr, Cu, Fe, Ir, Mn, Ni, Pt and many others, and allow potentially incorporation of an array of metals with different properties into nucleic acid and its components.

The electrochemical behavior of the cobalta- and ferradicarbollides $[(1,2\text{-}C_2B_9H_{11})_2M]^{n-}$ and their funcionalised derivatives is long known [11-13]. Cobaltacarboranes can shuttle the sequence CoIV/CoIII/CoII/CoI, whereas ferracarboranes can display the sequence FeIII/FeII/FeI, the different members

of the two sequences being more or less accessible as a function of the experimental conditions [14].

Nucleoside-metallacarborane conjugates with cobalt, iron or chromium for base- specific metal labeling.

Ring opening reaction in cyclic ethers and polyhedral boron hydride adducts with various nucleophiles has been established over last two decades as useful methodology for the attachment of functional groups on the boron cluster derivatives [15]. Taking the advantage of this approach we have developed a general method for the synthesis of metallacarborane derivatives containing cobalt of all four canonical nucleosides: thymidine, 2'-*O*-deoxyadenosine, 2'-*O*-deoxycitidine, and 2'-*O*-deoxyganosine [16].

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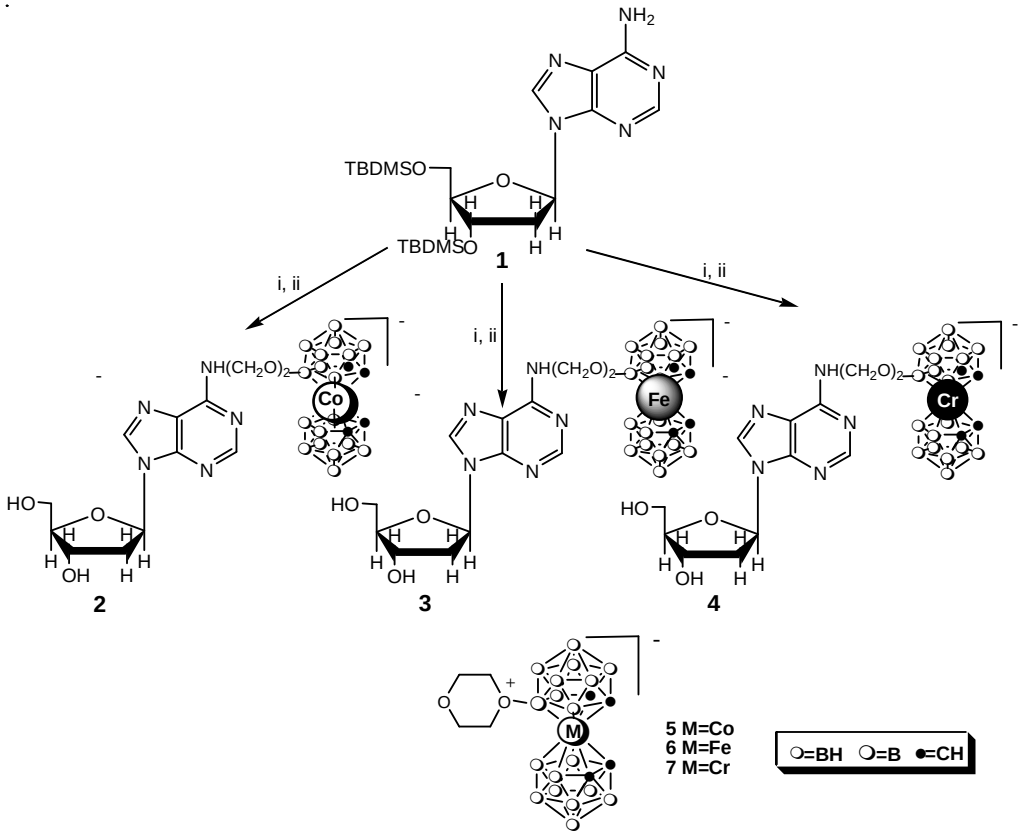


i. NaH/toluene,**5, 6** or **7**, 70^{0}C, ii. TBAF/THF; TBDMS=*tert*-butyldimethylsilyl group

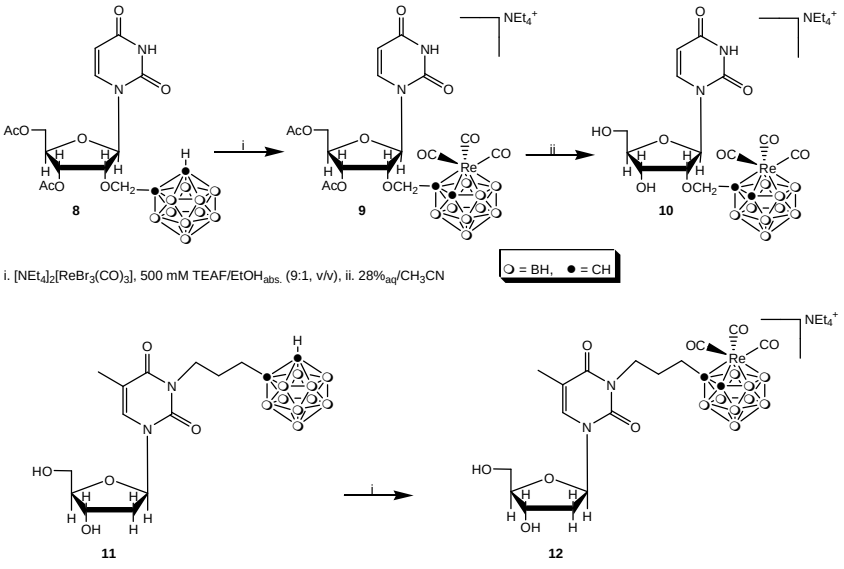
Scheme 1. Synthesis of 2'-deoxyadenosine modified with metallacarboranes containing cobalt, iron and chromium.

This method is one of the very few available approaches useful for direct synthesis of metal-complex containing nucleoside conjugates [7].

Versatility of the above methodology was demonstrated by the extension of the original approach towards synthesis of nucleoside conjugates bearing other than cobalt metals: iron [17] and chromium [18] (Scheme 1). Synthesis was performed analogously as described for [8-dioxane-3-cobalt bis(dicarbollide)]^0 adduct [16]. It is worthy to point out that the approach based on oxonium ring opening offers a route to nucleoside conjugates bearing any metal - as long as suitable cyclic ether/metallacarborane adduct is available. Work on expanding the range of metals available for nucleoside modification according to above methodology is ongoing in our laboratory.

Nucleoside-metallacarborane conjugates with rhenium.

Recently were developed a new method of the nucleoside-metallacarborane conjugates synthesis based on *de novo* formation of metallacarborane complex type of 3,3,3-tricarbonyl-3-metal-1,2-dicarba-*closo*-dodecaborate.



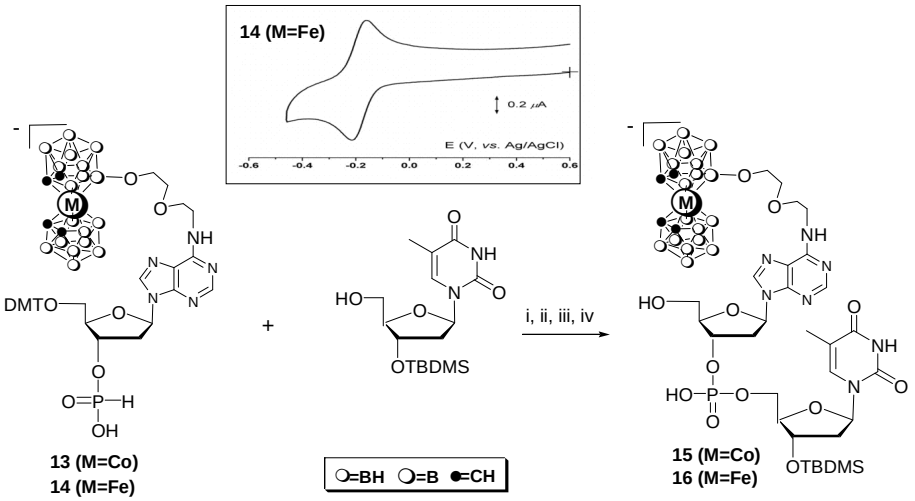
Scheme 2. Synthesis of nucleoside-metallacarborane conjugates containing rhenium (I).

An example procedure for the nucleoside conjugate bearing rhenium Re(I) is shown on Scheme 2 [19]. The reaction is performed analogously as described for synthesis of parent complex, 3,3,-tricarbonyl-3-rhenium-1,2-dicarba-*closo*-dodecaborate [20].

Briefly, an aqueous solution of 500 mM tetraethylammonium fluoride (TEAF) containing a small quantity of absolute ethanol, which was needed to solubilize the substrate, was used to prepare the appropriate *nido*-carborane salt from compound **15**, next reaction with slight excess of $[NEt_4]_2[Re(CO)_3Br_3]$ was performed. The heterogenous suspension, was kept at elevated temperature for several hours till TLC analysis indicated complete consumption of the substrate **15**. It is worthy to point out that reports on the direct formation of a metallacarborane from the corresponding *closo*-isomer of 1,2-dicarba-*closo*-dodecaborane are rare [21]. In addition, the proposed procedure consists a first example of this type reaction carried out in water as a reaction medium [20].

DNA-oligonucleotide bearing carborane and metallacarborane cluster.

Nucleosides/metallacarborane conjugates bearing iron and cobalt were used for the synthesis of short DNA-oligonucleotides. DNA-dimers have been obtained *via* H-phosphonate method in solution, in reaction of 5'-*O*-dimethoxytrityl H-phosphonate nucleotide component **13** or **14** bearing metallacarborane with 3'-*O*-(*tert*-butyldimethylsilyl)-thymidine. Fully protected dinucleotide intermediate was next oxidized to transform H-phosphonate internucleotide linkage into natural phosphate one yielding **15** and **16**. Subsequently, 3'-*O*-(*tert*-butyldimethylsilyl) group and 5'-*O*-dimethoxytrityl group has been removed with TBAF and 80% acetic acid, respectively, yielding final dinucleotide conjugate (Scheme 3).



i. PvCl/pyridine, ii. 10% $H_2O/CCl_4/Et_3N$/N- methylimidazole (9:0.5:0.5 v/v); iii. TBAF/THF, iv. 80% AcOH

Scheme 3. Dinucleotide/metallacarborane bearing iron and cobalt.

All isolated intermediates and final products were fully characterized by UV, IR, TLC, ^{1}H, ^{13}C, ^{11}B NMR spectroscopy, FAB mass spectroscopy [17]. Dinculeotide **16** (M=Fe) was also studied by cyclic voltammometry in DMF ($0.2x10^{-3}$ mol dm^{-3}) solution using a glassy carbon electrode and $[NEt_4][ClO_4]$ (0.1 mol dm^{-3}) as supporting electrolyte [22].

Automated, solid phase phosphoramidite method was developed for the synthesis of the oligonucleotides containing *o*-carboran-1-yl-cage attached to the 2'-position of uridine through the methylene bridge (2'-CBM-oligonucleotides) (Figure 1) [23].

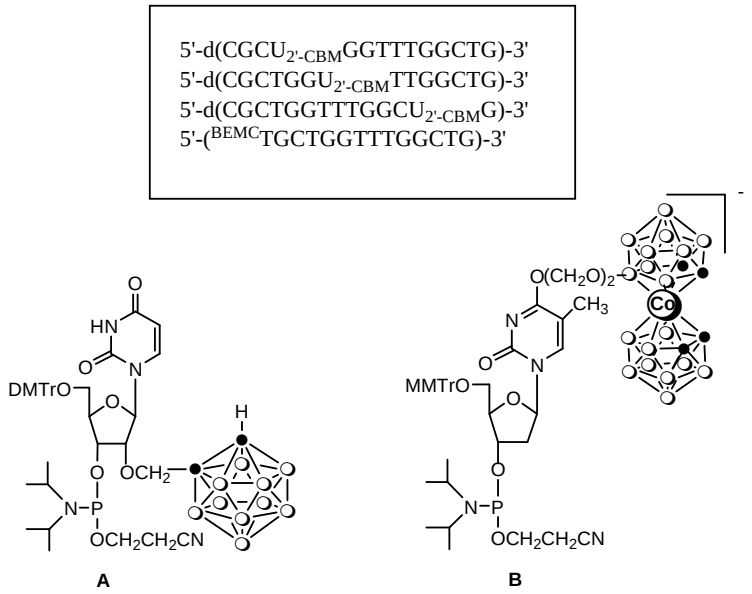


Figure 1. Sequence of DNA-oligonucleotide modified with carborane and metallacarborane group; monomers which were used for synthesis **A** (2'-CBM), **B** (BEMC).

Manual steps were performed for the insertion of modified monomer (Figure 1, **A**) bearing 2'-CBM group. All modified DNA-oligonucleotides were complementary to DNA-HCMV, and contained 2'-CBM modification near 3'-end, 5'-end or in the middle of the oligonucleotide chain. The resulting oligomers were characterized by reverse phase high-performance liquid chromatography (RP-HPLC) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) [23].

We have described also a method for the synthesis of metallacarborane containing oligonucleotides [10]. As the metallacarborane modified nucleoside

unit 4-*O*-diethylenoxy-8-[(1,2-dicarba-*closo*-undecaborane)-3,3'-cobalt-(1',2'-dicarba-*closo*-undecaborane)]thymidine (BEMC, isomer 4-*O*) was used. Its conversion into 5'-*O*-monomethoxytrityl-4-*O*-diethylenoxy-8-[(1,2-dicarba-*closo*-undecaborane)-3,3'-cobalt-(1',2'-dicarba-*closo*-undecaborane)]-3'-*O*-(*N*,*N*-diisopropyl-*β*-cyjanoethyl)phosphoramidite (Figure 1, **B**) provided modified monomer for the synthesis of BEMC-oligonucleotides [10]. Synthesis of metallacarborane-modified oligonucleotide was performed using a standard, automated, solid phase phosphoramidite procedure, with the exception that metallacarborane-modified nucleoside was attached manually to the 5'-end of the oligomer chain. The purity of oligonucleotide was checked by polyacrylamide gel electrophoresis (PAGE) and RP-HPLC; the integrity of the oligomer was confirmed by mass spectrometry.

Electrochemical properties of nucleoside conjugates containing metallacarborane complex of cobalt, iron, chromium.

Preliminary studies of electrochemical properties of 4-*O*-diethylenoxy-8-[(1,2-dicarba-*closo*-undecaborane)-3,3'-cobalt-(1',2'-dicarba-*closo*-undecaborane)]thymidine, were performed under HPLC conditions using RP-HPLC (Hewlett Packard 1050 and Altech Econosil C18, 5μm, 4.6x240 mm column) equipped with electrochemical detector Coulochem II (Esa, Inc., USA) and amperometric analytical cell Model 5040 (Esa, Inc., USA) (Figure 2) [24]. Intense diagnostic peak at R_t=4.8 min. was detected at potential +1.7 V (vs. palladium electrode) due to redox activity of the metallacarborane (Co(III)/Co(II)) (Figure 2) [25]. For unmodified thymidine no signal was detectable electrochemically.

6-*N*-Diethylenoxy-8-[(1,2-dicarba-*closo*-undecaborane)-3,3'chromium-(1',2'-dicarba-*closo*-undecaborane)]-2'-deoxyadenosine, a nucleoside/metallacarborane conjugate bearing chromium was tested under analogous condition. Intense diagnostic peak at R_t=7.1 min. was detected for **4** at potential +1.6 V (vs. palladium electrode) due to redox activity of the metallacarborane, (Cr(III)/Cr(II)) [18, 25]. To confirm identity of the observed electrochemical signal, HPLC experiments under the same conditions but with UV instead of electrochemical detection were run for **4** and 2'-deoxyadenosine.

Electroactivity of 2'-*O*-(7,8-dicarba-*nido*-undekaborane-7-ul)methyl-uridine bearing 7,8-dicarba-*nido*-undekaborate was also tested under RP-HPLC conditions analysis with electrochemical detection Coulochem II and coulometric analytical cell Model 5010 (Esa, Inc., USA) [23]. Intense diagnostic peak at R_t=4.6 min. was detected at potential +0.6 V (vs. Pd/H_2 electrode) (Figure 3). For unmodified uridine no signal was detectable electrochemically.

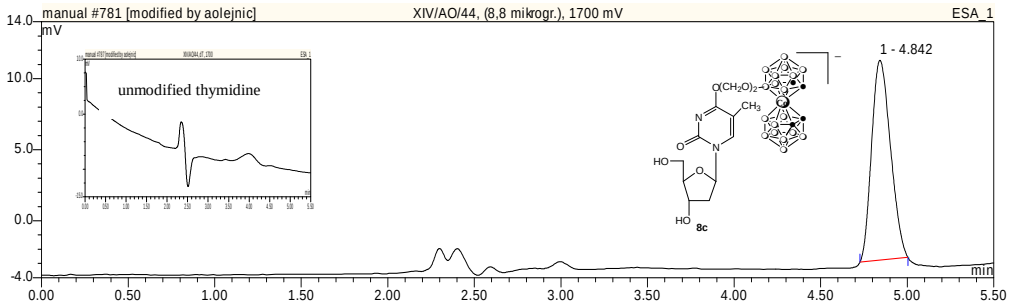


Figure 2. RP-HPLC analysis with electrochemical detection of metallacarborane/thymidine conjugate containing cobalt; picture inside: RP-HPLC analysis with electrochemical detection for unmodified thymidine.

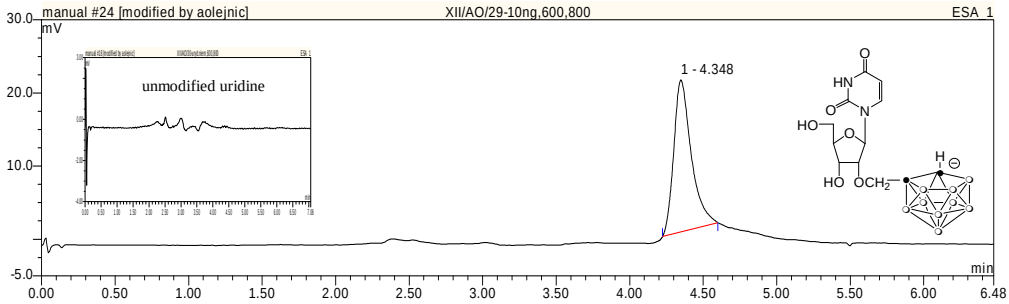


Figure 3. RP-HPLC analysis with electrochemical detection of 2'-*O*-(7,8-dicarba-*nido*-undekaborane-7-yl)methyluridine; picture inside: RP-HPLC analysis with electrochemical detection for unmodified uridine.

The conjugates of all four canonical nucleosides: thymidine, 2'-deoxycytidine, 2'-deoxyadenosine, 2'-deoxyguanosine and metallacarborane bearing cobalt also 2'-deoxyadenosiane and metallacarborane bearing iron and dinucleosides bearing cobalt and iron were tested more systematically by cyclic voltammetry [22].

At a glassy carbon as well as gold electrode, most of the Co(III) complexes exhibit two separate reduction processes featuring chemical reversibility in the cyclic voltammetric time scale (Table 1). The electrochemical tests have been carried out at T=-10^{o}C, in DMF solution containing [NEt_4][ClO_4] (0.1 mol dm^{-3}) as supporting electrolyte and Ag/AgCl reference electrode. All compounds with metallacarborane bearing cobalt exhibit two processes to the sequence Co(III)/Co(II)/Co(I). It is noticed that at a platinum electrode the second reduction is masked by the solvent discharge. No oxidation process was detected up to the anodic discharge of the DMF

solvent (≤+1.6 V). It was observed also that under the actual experimental conditions no well defined redox processes are exhibited by unmodified thymidine, 2'-deoxycytidine, 2'-deoxyguanosine, and 2'-deoxyadenosine. only in the case of 2'-deoxyguanosine a well defined, irreversible oxidation was detected (E_p=+1.17 V) which aggress with the known electrochemical activity of guanine base [6].

The conjugate of 2'-deoxyadenosine and metallacarborane bearing iron (Fe(III)) (**3**) exhibits only a single (coulometrically measured) one-electron reversible reduction. an irreversible oxidation occurs at high potential values (E_p=+1.43 V). Table 1 compiles the formal electrode potentials of the reversible redox changes exhibited by complex, together with those related derivatives.

Table 1. Formal electrode potentials (V, vs. Ag/AgCl) and peak-to-peak

Complex	$E°'_{M(III)/M(II)}$	ΔE_p [a]	$E°'_{M(II)/M(I)}$	ΔE_p [a]
2 (Co)	–1.13	88	–2.08	62
3 (Fe)	–0.07	69	-	-
T(Co) (isomer 2*O*)	–1.23	95	–2.18	72
T(Co) (izomer 3*N*)	–1.23	108	–2.20[b]	-
T(Co) (isomer 4*O*)	–1.19	74	–2.16	84
dC (Co) (4*N*)	–1.14	100	–2.12	92
dG (Co) (isomer 1*N*)	–1.02	83	–1.97	105
dG (Co) (isomer 2*N*)	–1.05	110	–1.99	108
dG (Co) (isomer 6*O*)	–1.05	74	–2.03	75
Dinucleoside (dA^{Co}-T) (15)	–1.05	73	–1.92[b]	-
Dinucleoside (dA^{Fe}-T) (16)	–0.19	64	-	-
[(4-OH-2,3-$C_2B_9H_{10}$)Co(2,3-$C_2B_9H_{11}$)]⁻	–1.20	94	–2.07	92
[4-($O(CH_2)_2O(CH_2)_2OH$)-2,3-$C_2B_9H_{10}$)Co (1,2-$C_2B_9H_{11}$)]⁻	–1.13	104	–2.08	78
[(2,3-$C_2B_9H_{11}$)$_2$Fe]⁻	–0.16	69	-	-

[a] Measured at 0.2 Vs^{-1}; [b] from Osteryoung square wave voltammetry because of the presence of a partially overlapping spurious peak

It is evident that the different bio-active appendices play slight but significant inductive effects, for example for three isomers of thymidine. Substitution of 2'-deoxycytidine or 2'-deoxyadenosine has no substantial effect on redox potential of metallacarborane component.

Electrochemical DNA detection using carborane cluster label.

Applicability of 7,8-dicarba-*nido*-undekaborane-7-yl group (CBM) as a DNA redox label was tested. Under HPLC conditions electrochemical detector Coulochem II (Esa, Inc., USA)) DNA-oligonucleotide probe complementary to a fragment of US14 gene of human cytomegalovirus (HCMV) labeled with CBM group, provided an intense diagnostic peak at potential +0.9 V at carbon electrode (vs Pd/H_2) (Figure 4) [23].

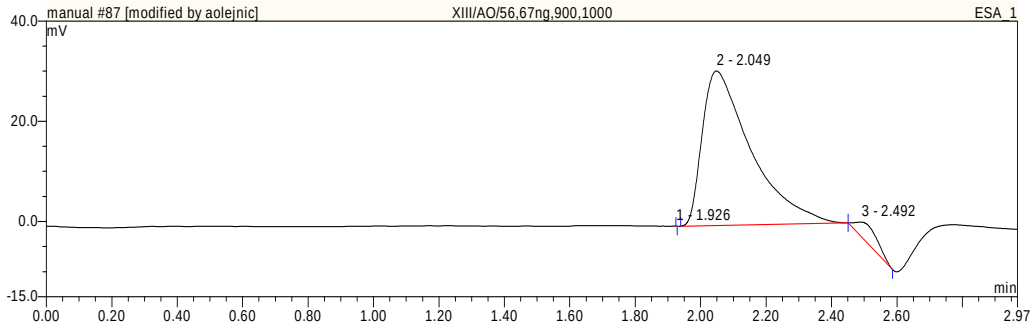


Figure 4. RP-HPLC analysis of 5'-d(*CGCTGGTTTGGC(*$U_{2'\text{-}CBM}$*)G*)-3' (R_t=2.05 min.)

On the base of the above results a new electrochemical DNA sensor for the detection of labeled oligonucleotide probe by means of the CBM group as electroactive redox-marker was proposed [27].

Differential-pulse or square-wave voltammetry in combination with an adsorptive transfer stripping technique was utilized for measuring of ODN hybridization. The whole procedure involves ODN hybridization at superparamagnetic Dynabeads (DB) with subsequent detection at the surface of a carbon paste electrode. Schematic representation of electrochemical detection of ODN hybridization between probe and target ODN is shown in the Figure 5.

Nanomolar concentration of CBM-labeled target ODN was recognized at carbon paste electrodes. Further increase in sensitivity is possible by incorporation of more than one boron cluster label into the DNA-oligonucleotide probe [28].

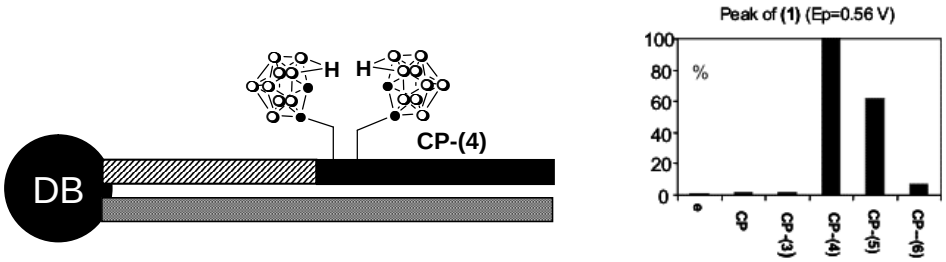


Figure 5. Electrochemical detection of unlabeled (**CP** and **3**) and boron cluster labeled DNA-oligonucleotide probes (**4**-**6**).

The above finding, together with proposed earlier use of metallacarboranes as electrochemical label for biomeolecules [22] lays the foundations for a "multi color" electrochemical coding of DNA with boron clusters and simultaneous detection of several DNA targets. The only recently published synthesis and electrochemical tuning of dodecaborate derivatives with pseudometallic properties [29] expands the potential of boron clusters as entirely new, flexible and adjustable electrochemical labels for biomolecules.

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1,2-DICARBADODECABORANES: CHEMICAL STRATEGIES FOR DELIVERY AND OPPORTUNITIES AS LIGAND PLATFORMS

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Summary

The synthetic medicinal chemistry group at the University of Bath has, during the past 15 years, attempted to address two of the outstanding issues in tumour-selective delivery of boron clusters for BNCT, tumour-targetting and aqueous solubility. Syntheses of 1,2-dicarba-*closo*-dodecaboranes have been linked to nitroimidazoles, nitrofurans, porphyrins and DNA-groove-binding PBDs have been achieved. Increased polarity of the targetting group carborane constructs was accomplished without charge through $(CH_2CH_2O)_n$ units attached either at the periphery of the construct or as part of the linker joining the modules of the construct. A carborane–β-cyclodextrin inclusion complex with remarkable stability has been characterised; this stability may shed some light on the deleterious effect of cyclodextrins in solubilising carboranes in biological experiments. A simple cage degradation of 1,2-dicarba*closo*dodecaboranes to corresponding *nido*-carboranes has been developed, which may facilitate an important non-BNCT use of icosahedral carboranes as neutral scaffolds or platforms with unusual geometry for conformationally controlled display of bioligands.

Key words: 1,2-Dicarbadodecaborane, hypoxia, ^{11}B NMR, porphyrin, ligand platform

Introduction

Boron neutron capture therapy (BNCT) is under active investigation for the treatment of various cancers [1,2]. Early clinical failures were attributed [3,4] to inadequate concentrations of ^{10}B in the tumour tissue or to lack of selectivity of disposition of ^{10}B, leading to damage to normal tissue. Thus one of the remaining major issues in BNCT is the development of water-soluble boron-containing drugs that are selectively taken up or retained by tumours. Biologically stable boron clusters, including icosahedral carboranes, have been linked to a variety of chemical structures in attempts to target boron selectively to tumours [5-8]. Since the neutral carboranes and several of the tumour-targetting moieties are highly lipophilic, solubility in water has also become an issue in the design of agents which deliver ^{10}B selectively to tumours.

This review summarises the medicinal chemistry carried out at Bath over the past 15 years to address the attachment of the 1,2-dicarba*closo*decaborane(12) cluster to tumour targetting moieties and approaches to increasing the aqueous solubility of the carborane-targetting group constructs and outline prospects for a non-BNCT application of this neutral icosahedral cluster.

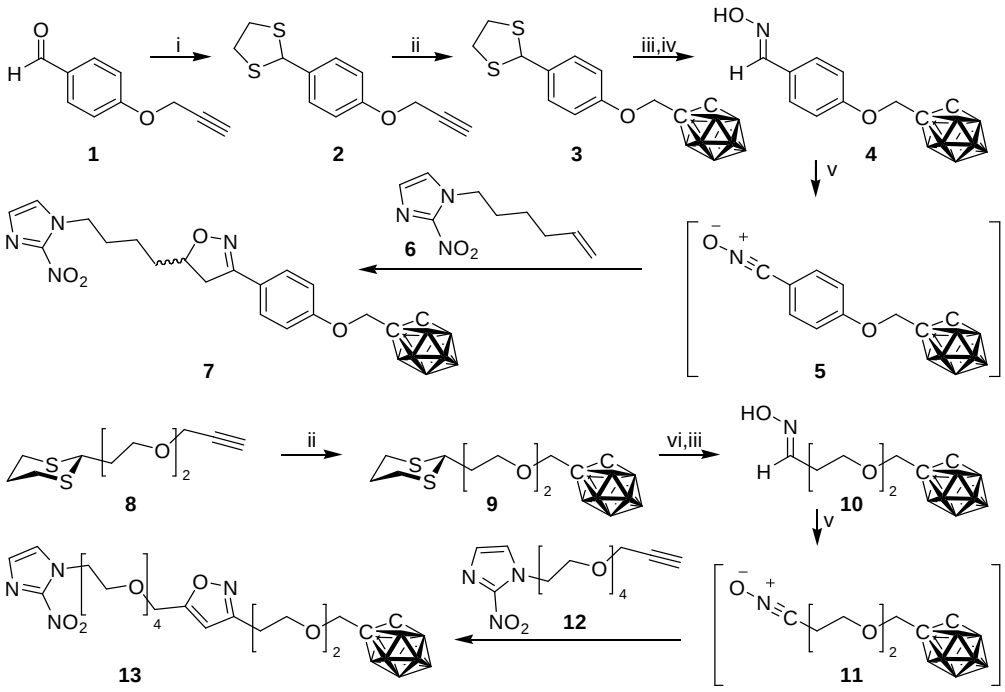
Discussion

Nitroimidazole–carboranes

Solid tumours >1 mm in diameter contain regions of chronic or acute hypoxia, owing to poorly organised or poorly structured tumour vasculature. For example, blood vessels in tumours can have closed ends but often have poorly developed walls, leading to collapse of the vessel under the high interstitial pressure found in tumours. Indeed, the poor structure of the vessel walls makes them leaky and contributes to this high pressure. Cells in hypoxic regions of solid tumours are relatively resistant to radiotherapy, since damage to target DNA requires the participation of O_2. Similarly, these hypoxic cells are killed less efficiently by conventional antiproliferative chemotherapy, as these cells do not receive sufficient O_2 and other nutrients to permit proliferation; moreover, induction of the hypoxia-stress response leads to increased cell survival.

1-Substituted 2-nitroimidazoles were developed in the 1970s and 1980s as electron-affinic radiosensitising drugs, which mimic the action of O_2, and as hypoxia-activated prodrugs to generate cytotoxins in these difficult tumour cells [9,10]. These compounds proved to be selectively retained in hypoxic tumour tissue through reductive metabolism to electrophiles [11,12]. In view of this selective retention in hypoxic tumour cells, we rationalised that attaching boron clusters to 2-nitroimidazoles might prove to be an effective way of achieving concentrations of ^{10}B in tumours, higher that those in surrounding normal tissues. The cluster that attached, through a linker, to the 1-position of the nitroheterocycle was 1,2-dicarba-*closo*-dodecaborane(12), a biostable cluster. This cluster and the nitroimidazole are highly liopohilic, so careful design of the linker was required to incorporate water-solubilising ($(CH_2CH_2O)_n$) units.

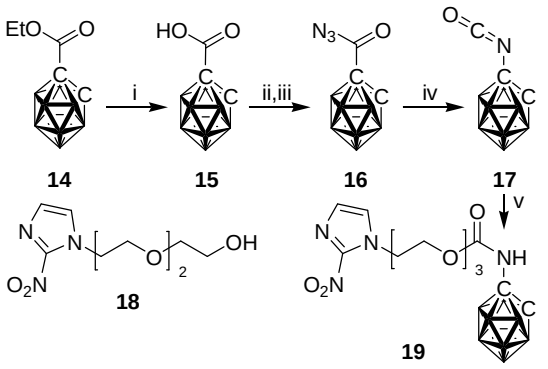
One issue in designing the synthesis of the nitroimidazole–carboranes was that the nitroimidazole is highly electron-affinic (E^1_7 = -389 mV), whereas the carborane is potentially a hydride donor. Thus very mild conditions were required for the final step of joining the nitroimidazole unit to the carborane unit. Two reactions were investigated for this final step, 1,3-dipolar cycloadditions of nitrile oxides with terminal alkenes and alkynes and formation of a carbamate from an isocyanate and an alcohol, both atom-efficient reactions.



Scheme 1. Syntheses of exemplary nitroimidazole-carboranes **7** and **13**. *Reagents*: i, $(CH_2SH)_2$, $BF_3.Et_2O$; ii, $B_{10}H_{14}$, MeCN; iii, $Hg(ClO_4)_2$; iv, $H_2NOH.HCl$, Na_2CO_3; v, NaOCl, CH_2Cl_2, H_2O; vi, NBS, $AgNO_3$, H_2O, MeCN. Unmarked vertices on the icosahedra are BH.

Nitrile oxides are most readily prepared by chlorination of aldoximes, followed by elimination of HCl. Thus the challenge was to prepare a carborane carrying an aldehyde moiety. A simple model example, carrying a benzaldoxime, was synthesised as shown in Scheme 1. To withstand the vigorous conditions under which the carborane is formed, the aldehyde of **1** had to be protected as the dithioacetal **2**; other protections for this group were destroyed by the Lewis acidity and hydride donation of the $B_{10}H_{14}$ in the following step. The carborane **3** was formed in the usual way, with $B_{10}H_{14}$ in the presence of a Lewis base. Hg^{2+}-catalysed deprotection revealed the benzaldehyde, which was converted to its oxime **4**. The most expeditious method to generate **5** was oxidation with aq. bleach; cycloaddition with the nitroimidazole alkene **6** formed the first nitroimidazole–carborane **7** [13,14]. This construct **7** was prepared to demonstrate that the linking chemistry was feasible and compatible with both the nitroimidazole and the carborane; it lacks water-solubilising features and is highly hydrophobic. Scheme 1 (lower) shows the adaptation of this synthetic route to the preparation of a nitroimidazole-carborane **13** where the linker contains six ether oxygens in $((CH_2CH_2O)_n)$ motifs (analogous to the highly water-soluble PEGs) and lacks the hydrophobic benzene. An aliphatic aldehyde was again introduced as a dithioacetal, in **8**.

Formation of carborane **9** was followed by deprotection (now needing S-bromination) and conversion to oxime **10**. The NaOCl / water / CH_2Cl_2 system again generated the carborane–nitrile oxide **11** efficiently for reaction *in situ* with the nitroimidazole–alkyne **12** to afford the target nitroimidazole-carborane **13**. This chemistry was then exploited to generate a series of nitroimidazole–carboranes with isoxazole links and $(CH_2CH_2O)_n$ units in both arms [15]. Removal of the carborane 2-H and quench with HO^3H gave a ^3H-labelled isotopomer [16].



Scheme 2. Synthesis of carbamate-linked nitroimidazole-carborane **19**. *Reagents*: i, NaOH, aq. EtOH; ii, $SOCl_2$; iii, Me_3SiN_3; iv, PhMe Δ; v, **18**.

A short series of carbamate-linked nitroimidazole–carboranes was also prepared; these were designed to replace the lipophilicity of the central isoxazole of the above compounds with a polar carbamate (example: Scheme 2) [16]. Curtius rearrangement of the carborane–acyl azide **16** gave the isocyanate **17**, which reacted *in situ* with the nitroimidazole–alcohol **18** to give the nitroimidazole–carborane **19**, which also carries $(CH_2CH_2O)_n$ for increased solubility.

The selective retention of two nitroimidazole–carboranes in experimental solid tumours was examined spectroscopically [17]. Samples of **13** and **19** containing boron at the natural isotopic ratio were administered *i.p.* to female C_3H mice carrying subcutaneous SCCVII/Ha or KHT tumours, respectively, at doses of 0.81 mmol Kg^{-1}. ^{11}B NMR spectra were obtained using a surface coil in a 4.7 T horizontal bore magnet (Figure 1). For comparison, Figure 1 also shows ^{11}B spectra of **18** in a conventional spinning 5 mm NMR tube and in a 10 mL round-bottom flask (used as a phantom to set up the *in vivo* experiments). These spectra indicate that there is some selective retention of ^{11}B from **19** in the tumour after 24 h, whereas it had washed out of a normal tissue, brain, at that time. However, much material accumulated in liver from **19**; the (undesired) uptake of **13** into liver was much lower.

Other hypoxia-activated carborane prodrugs

Another approach to hypoxia-selective retention of drugs, including boron clusters, is to design a protecting group, which is removed by (bio)reduction selectively in hypoxic tumours cells. An example of this type of prodrug was constructed (Scheme 3). Hydrolysis of the cyanopropylcarborane **20**, followed by formation of the acyl chloride and treatment with NaN_3 gave

the acyl azide **21**. Curtius chemistry again led to the isocyanate **22**, which reacted readily with 5-nitrofuran-2-methanol to provide the candidate prodrug **23**. The release of the aminopropylcarborane **25** was examined, using several reductants selective for the nitro group. Each caused very rapid release of **25**, once the nitrofuran of **23** had been reduced to the aminofuran of **24**; the increased electron density in the heterocycle then forced expulsion of the carborane-containing leaving group [19].

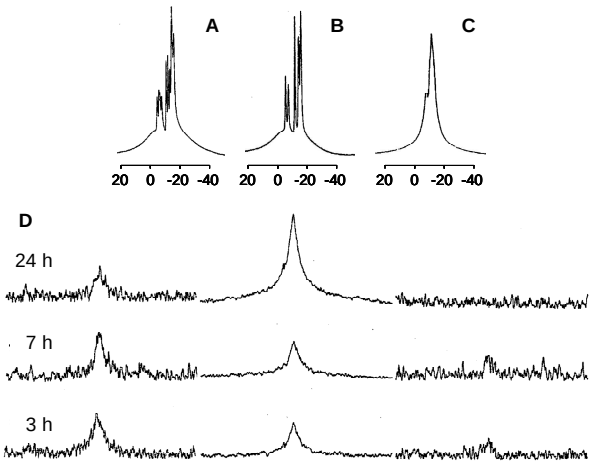


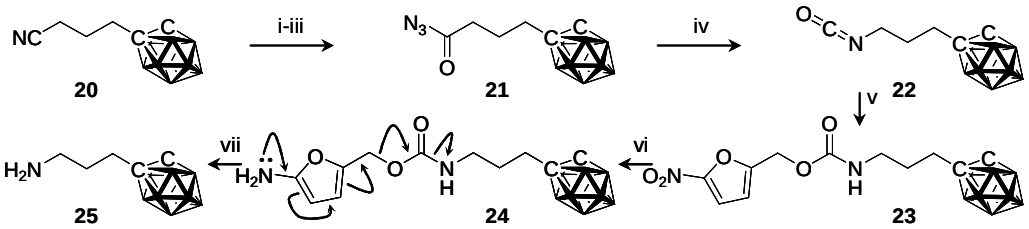
Figure 1. A. *In vitro* ^{11}B NMR spectrum of a solution of **19** in a spinning 5 mm tube at 9.4 T. **B.** *In vitro* ^{11}B spectrum (^{1}H-decoupled) of **19** in a spinning 5 mm tube at 9.4 T. **C.** *In vitro* ^{11}B spectrum of a solution of **19** in a non-spinning 10 mL RB flask in a 4.7 T horizontal bore instrument. **D.** *In vivo* ^{11}B spectra from SCCVII/Ha tumour, liver and brain in a C_3H mouse, 3, 7 and 24 h after administration of **19** (0.81 mmol Kg^{-1}). The vertical scale for the liver spectra is 0.2× that for the tumour and brain spectra.

Porphyrin–carboranes

Selective accumulation of porphyrins in tumours was first observed in the 1940s [20]; the use of porphyrins as boron carriers for BNCT arose from PDT studies by Dougherty [21]. Since then, several groups have prepared porphyrins carrying boron and have evaluated them in BNCT. The early boron-carrying porphyrins were β-substituted *meso*-free derivatives of haem. In these, water-solubility is achieved through carboxylates and, in the latter, by cage degradation of the 1,2-dicarba-*closo*dodecaboranes to anionic *nido* clusters. There has recently been considerable development work on generating porphyrins carrying many B atoms (up to eighty [22]), while employing various strategies to increase solubility and decrease phototoxicity (reviewed (1)). Generally, the *meso*-tetraphenylporphyrins (TPPs) carrying boron clusters at the *meso*-Ar groups present less problems of toxicity than do the haem derivatives and have been most successful in biodistribution studies *in vivo*.

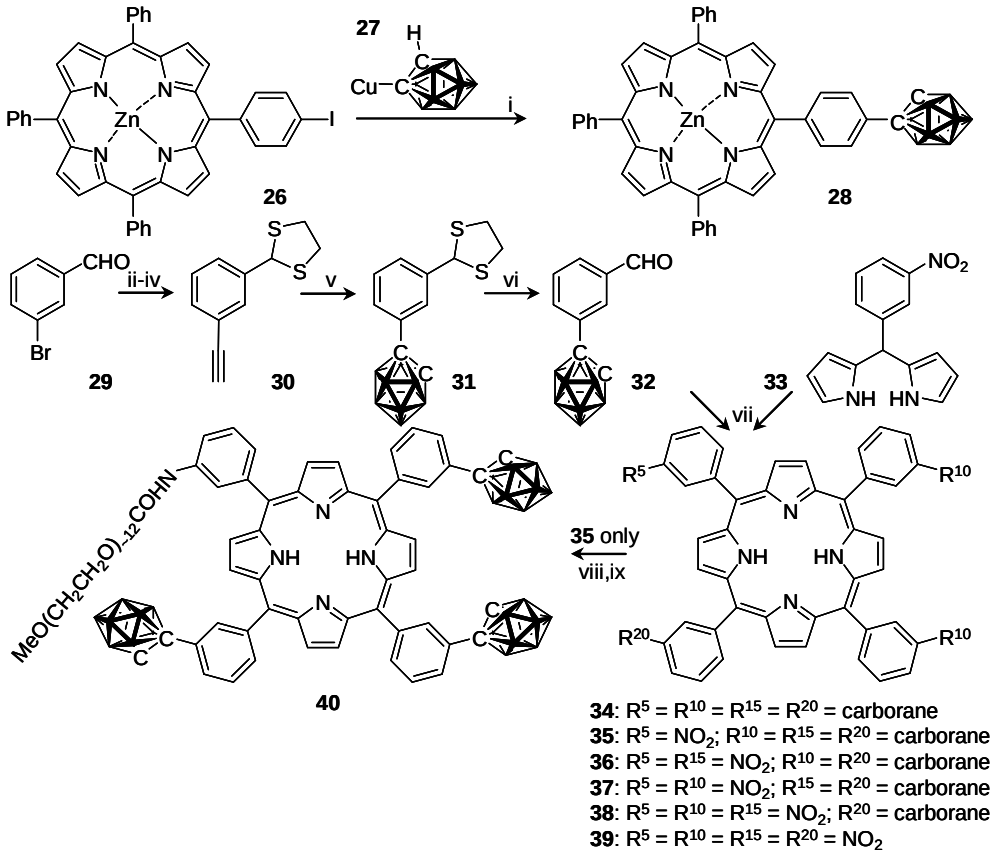
Most reported porphyrin–carboranes have the carborane linked to the porphyrin core through ethers or amides, both of which are potential targets for metabolic cleavage. Most also rely on their anionic nature for solubility; this may adversely influence their intracellular biodistribution. We sought to generate porphyrin–carborane constructs in which the carboranes have all-carbon links to the core and which are electrically neutral. Our initial approach was to develop a method by which carboranes could be coupled to the phenyls of a

TPP core as the final step of the synthesis, to maximise yields based on expensive ^{10}B. Coult *et al*. reported an interesting coupling of iodoarenes with 1,2-dicarba*closo*dodecaboranato-copper(I) species to give 1-arylcarboranes in high yields [23]. However, the maximum yield of the mono-carboranylporphyrin **28** from the coupling of the Cu(I)-carborane **27** with the monoiodophenylporphyrin **26** was 6%, achieved after 12 d at reflux in diglyme (Scheme 4) [24].



Scheme 3. Synthesis of nitrofuran-carborane **23** and reductively-triggered release of carborane-amine **25**. *Reagents*: i, H_2SO_4, H_2O, Δ; ii, $SOCl_2$; iii, NaN_3; iv, $CHCl_3$, Δ; v, 5-nitrofuran-2-methanol; vi, $NaBH_4$, Pd/C, Pr^iOH or $SnCl_2$.

As final-step couplings of this type appeared to be impracticable, a method was developed in which the porphyrin core was assembled from subunits carrying carboranes. These syntheses (Scheme 4) show one route to porphyrins carrying both carboranes and potentially water-solubilising poly(oxyethylene) units. We sought to exploit the "non-scrambling" conditions for regiocontrolled assembly of TPPs carrying different Ar-substituents claimed by Lee and Lindsey [25]. Sonogashira coupling of trimethylsilylethyne with 3-bromobenzaldehyde **29**, followed by desilylation and protection of the aldehyde, as usual, as a dithioacetal gave **30**, ready for formation of the carborane **31**. Hg^{2+}-catalysed deprotection afforded the carboranylbenzaldehyde **32**. However, condensation of **32** with the nitrophenyldipyrromethane **33** gave only a statistical mixture of the scrambled porphyrins **34**-**39** carrying 0-4 carboranes and 4-0 nitro groups, rather than the intended pure dicarboranyl-dinitro compound **36** [26]. Porphyrins **34**-**39** were all separable chromatographically; interestingly, the regioisomers **36** and **37** gave identical ^1H NMR spectra, owing to their symmetry, and could only be distinguished by an X-ray crystal structure of the original target **36**. The nitro groups had been incorporated into the design of the compounds as potential points of attachment of solubilising groups, after reduction. Reduction of the single nitro group of **35**, followed by acylation with the chloroformate derived from a polydisperse monomethyl-PEG, afforded porphyrin **40**, which carries thirty boron atoms and a $(CH_2CH_2O)_n$ chain [26]. Similar constructs were built with ether links between carboranes and phenyl rings.

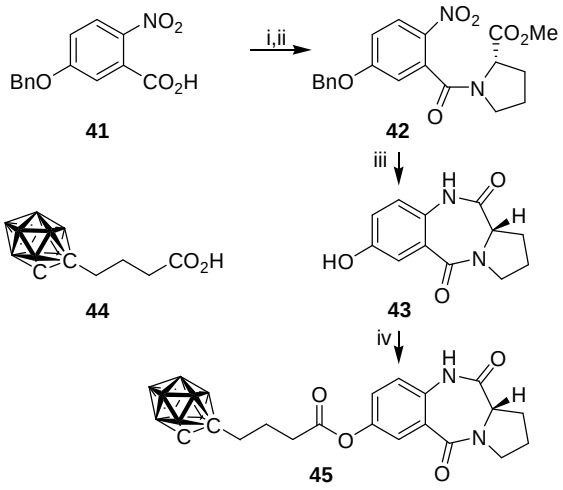


Scheme 4. Synthetic approaches to carbon-linked porphyrin carboranes. *Reagents*: i, diglyme, pyridine, Δ, 12 d; ii, $Me_3SiC{\equiv}CH$, Et_3N, $Pd(OAc)_2$, PPh_3; iii, K_2CO_3, MeOH; iv, $(CH_2SH)_2$, $BF_3.Et_2O$; v, $B_{10}H_{14}$, MeCN; vi, $Hg(ClO_4)_2$; vii, $BF_3.Et_2O$, CH_2Cl_2 then DDQ; viii, $SnCl_2$, AcOH; ix, Et_3N, $MeO(CH_2CH_2O)_{\sim 12}COCl$, DMAP.

PBD dione–carboranes

Pyrrolo[2,1-*c*][1,4]benzodiazepine-5-ones (PBDs) have been recognised for many years as very potent cytotoxins which bind in the minor groove of DNA and react covalently there [27,28]. However, the closely analogous pyrrolo[2,1-*c*] [1,4]benzodiazepine-5,11-diones (PBD diones) have more recently been recognised [29,30] as weaker and non-covalent ligands for the DNA minor groove. Thus we predicted that attaching a carborane to the 7-position of a PBD dione through a short tether would present the boron at or near the outer rims of the DNA minor groove for more effective BNCT though the increased solid angle subtended by the DNA, the target for the damaging α-particle. Scheme 5 shows our approach to an example where the carborane is linked by an ester to the PBD dione. 2-Nitro-5-benzyloxybenzoic acid **41** was converted to its acyl chloride for coupling to L-ProOMe, to give **42**. Hydrogenolysis both reduced the nitro group and removed the benzyl protection; the newly generated NH_2 cyclised *in situ* to provide the tricycle **43** with the 7-

OH group. DCC coupling with the carboranylbutanoic acid **44**, formedation the ester in the target **45**, providing this first PBD-carborane derivative for BNCT studies and as a bulky lipophilic ligand to probe the structure and dynamics of the DNA minor groove.



Scheme 5. Synthesis of ester-linked PBD dione-carborane **45**. *Reagents*: i, $(COCl)_2$; ii, L-ProOMe, DMAP; H_2, Pd/C; iv, **44**, DCC, HOBt.

Cyclodextrin–carboranes

Attachment of carboranes to nitroimidazoles, porphyrins and PBD diones was designed to respond to the need for selective targetting of boron to tumours and to the DNA within the tumour cell; the issue of aqueous solubility remained, although it had been addressed in part by the poly(oxyethylene) units in selected nitroimidazole–carboranes and porphyrin–carboranes. Another approach investigated by us was complexation of the carborane with cyclodextrins (cyclic 1,4α-linked oligomers of D-glucose). These have often been used to solubilise lipophilic drugs by forming complexes in which aromatic groups are included within the cyclodextrin. However, when carboranylalanine was solubilised by a derivative of β-cyclodextrin, uptake into cells was actually inhibited by this complexation [31], an observation consistent with formation of a very strong complex between the cyclodextrin and the carborane. However, prior to our work, there was only one chemical study of inclusion complexes of icosahedral carboranes with cyclodextrins, in which complexes of α-, β- and γ-cyclodextrins with unsubstituted 1,2-dicarba*closo*dodecaborane(12) were isolated [32]; the stoichiometries were determined by elemental analysis and by integration of the ^{1}H NMR spectra but structures were speculative only.

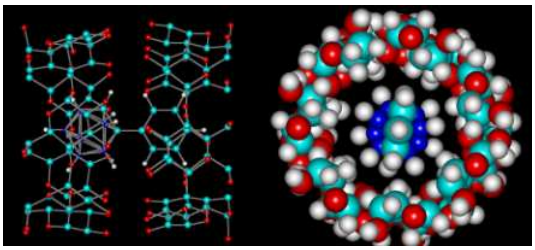
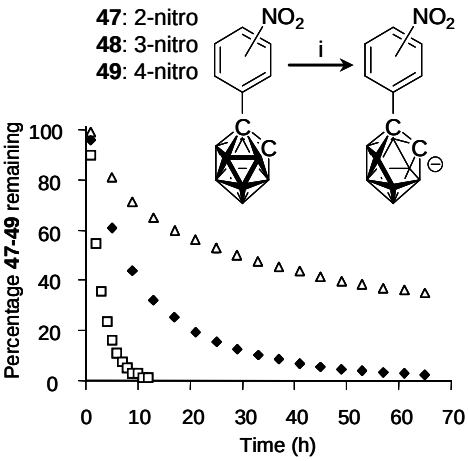


Figure 2. Structure of the 1:2 phenylcarborane: β-cyclodextrin complex **46** in side and plan views, derived from the modelling study.

To study the complexation of a simple carborane with cyclodextrins, 1-phenyl-1,2-dicarba*closo*dodecaborane was heated with two equivalents of β-cyclodextrin at reflux in aq. propan-2-ol. The precipitate obtained was washed

with hot water (to remove excess cyclodextrin) and hot toluene (to remove uncomplexed phenylcarborane), with no evidence of decomplexation. Interestingly, the complex was unchanged and unmelted by brief heating to 350°C (phenylcarborane melts at 65°C and uncomplexed β-cyclodextrin is caramelised by heating >200°C); thus the complex is extremely stable. It was characterised initially as a 1:2 carborane:cyclodextrin complex by integration of the ^{1}H NMR signals. There were no significant changes in the ^{1}H NMR chemical shifts of the protons in either component caused by the complexation. The structure was resolved by NOE (400 MHz) and NOESY (600 MHz) spectroscopy, with intermolecular NOEs between the carborane 2-H and inner protons of the cyclodextrin and between phenyl ring protons and inner protons of the cyclodextrin, showing that both the carborane and the phenyl were located within cyclodextrin cavities. Detailed analysis of the NOE patterns confirmed the structure as **46** (Figure 2), with tight complexation of the carborane and the two cyclodextrins meeting at their wide apertures [33]. A modelling study indicated that this arrangement of the cyclodextrins would allow the formation of a H-bond zipper around the circular interface of the cyclodextrins. This modelling also confirmed the carborane to be a very snug fit in the β-cyclodextrin cavity, providing an explanation for the refractory behaviour of this 1:2 complex and for the apparent excessive stability of carborane–β-cyclodextrin complexes in biological media.



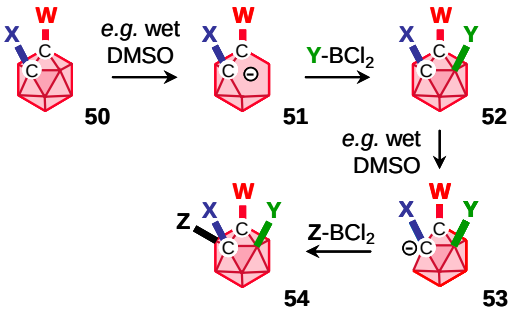
Scheme 6. Cage degradation of nitrophenyl-*closo*carboranes to the *nido* derivatives in wet $(CD_3)_2SO$ at 20°C. **47**:□ **48**:△ **49**:◆

*Cage degradation in 1-aryl-1,2-dicarba*closo*dodecaboranes.*

An approach often used to increase water-solubility of carborane-containing BNCT agents is to degrade the neutral *closo*-carborane cage to an anionic *nido*-carborane [34,35]. The most common method to achieve this deboronation is hydroxide ion at elevated temperatures [36] but amines [35,37] and fluoride [38,39] are also used. The disadvantage with these methods is that harsh conditions are often used, to the detriment of other groups.

During other studies on 1-aryl-1,2-dicarba*closo*dodecaboranes, we observed that the ^{1}H NMR spectrum of a sample of 1-(4-nitrophenyl)-1,2-dicarba-*closo*dodecaborane in wet $(CD_3)_2SO$ changed with time. Further investigation (^{1}H NMR, MS) revealed that deboronation was occurring to give an anionic

nido structure; a 2-D ^{11}B-^{11}B COSY NMR spectrum showed that 3-B (symmetrical with 6-B) was being lost; the is the same boron as is lost with the other cage degradation methods. The lost boron atom was converted to boric acid, as shown by the ^{11}B NMR spectra and acidification of the reaction medium. H_2 was evolved from the solution. Expansion of the NMR studies to other 1-(substituted aryl)-1,2-dicarba*closo*dodecaboranes showed that these also were degraded to the *nido* analogues under the same conditions [40]. All deboronations proceeded initially by first-order kinetics, as shewn by ^1H NMR kinetic studies (Scheme 6). Moreover, the rates rank correlated with the electron-withdrawing nature of the substituents, as indicated by their Hammett σ values, showing build-up of negative charge in the rate-determining step. This new mild deboronation, which had been noted previously for very highly electron-deficient 1,2-dicarba*closo*dodecaboranes [41-43], is extended to other 1-aryl-1,2-dicarba*closo*dodecaboranes and may be new tool in generating *nido*-carboranes for BNCT and for other chemical and biological applications.



Scheme 7. Schematic of proposed use of 1,2-dicarba*closo*dodecaboranes and platforms to display up to four (bio)ligands in defined conformations.

*Prospects for 1,2-dicarba*closo*-dodecaboranes as ligand platforms*

Protein–protein and carbohydrate–protein interactions are critical in many cellular processes. Interactions between a single amino-acid or a single sugar ligand and a protein are often weak but multiple amino-acids (in a peptide) or multiple sugars participate in binding to enhance the affinity and selectivity. For sugars, this is known as the glycosidic cluster effect [44]. To exploit this effect, a number of molecular scaffolds or ligand platforms have been develop which present amino-acids, short peptides or sugars in defined fixed orientations in space for binding to a target protein; these include calixarenes [45], cyclodextrins [46], cucubiturils [47], oligosaccharides [48] and steroids [49].

The icosahedron of the 1,2-dicarba*closo*dodecaboranes is unusual in organic chemistry. In particular, the two carbon atoms and the adjacent borons at positions 3 and 6 form two equilateral triangles with a common side. Attachment of individual amino-acids, short peptides or sugars to these vertices would present these ligands in a geometry fixed by the surface of the carborane. The facile deboronation chemistry above could readily be exploited to remove one boron atom from a 1,2-disubstituted 1,2-dicarba*closo*dodecaborane **50** (Scheme 7). The missing vertex of the icosahedron of *nido*carborane **51** can

be filled by reaction of the *nido*-carborane with an alkyl- or aryl-boron dichloride (**Y**BCl_2). The overall effect of the removal of the BH) and replacement with a boron carrying a substituent is to add the substituent to the *closo*-carborane specifically at boron-3. This generates an icosahedron with potentially three different groups at the corners of a surface triangle; this is chiral and could be resolved. Repetition of the deboronation/re-filling sequence could form an icosahedron **54** with four different groups. The concept is shown in cartoon form in Scheme 7. All four points of the array are potentially independently addressable; groups **W** and **X** are introduced by conventional carborane chemistry, whereas **Y** and **Z** are added through re-filling vacant vertices with boron dichlorides. Careful selection of these groups could allow different peptides or sugars to be attached to each, to generate a range of conformationally controlled biological probes.

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THE CARBORANE CLUSTER IN COMUTATIONAL DRUG DESIGN

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Summary

The docking of *closo*- and *nido*-carboranyl antifolates into human dihydrofolate reductase (hDHFR) was investigated with Autodock 4. The cage structures of these antifolates bound to a hydrophobic pocket within the active site of the hDHFR crystal structure through van der Waals interactions. The docked poses of the antifolates were similar to those of the corresponding ligand poses in the original hDHFR crystal structure. Minor variations in partial atom charges and cage geometries had no significant impact on docking accuracy. The obtained docking patterns validated methods that were previously developed for the computational design of carborane-containing compounds.

Key Words: Carboranyl Antifolates, Docking, Human Dihydrofolate Reductase (hDHFR)

Introduction

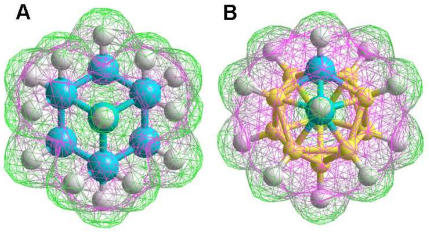

Figure 1: Dimensions and isosurfaces of adamantane (A) and *o*-carborane (B). Structures were build minimized (MM+)

Chemical, physicochemical, and structural versatility combined with high stability under physiological conditions are distinctive features of carboranes and other boron clusters [1-4] they have been used for decades in the design and synthesis of therapeutics for Boron Neutron Capture Therapy (BNCT) of cancer and, more recently, also in other areas of drug design [1-3]. Hydrophobic *closo*-carboranes, comparable in dimensions to adamantane (**Fig. 1**), were used as bioisosteric replacements for (hetero)aromatic and (hetero)aliphatic ring systems and other bulky entities in the design and synthesis of carboranyl derivatives of various amino acids and peptides,(3) estrogen receptor agonists/antagonists [5-9], androgen receptor antagonists [10-12], cholesterol

[13], retinoids [14,15], benzolactamic protein kinase C inhibitors [16], thalidomide [17], flufenamic acid and diflunisal [18], thrombin inhibitors [19], and trimethoprim [20]. Many of these boronated derivaties displayed biological activities comparable or even superior to those of their non-boronated counterparts. Also, metallocarboranes were found to be effective inhibitors of HIV-1 protease [21].

Negatively charged boron cluster also have been utilized as “prosthetic groups” for radiohalogens in the design and synthesis of radiotherapeutics and imaging agents [22-25]. They are readily halogenated and the boron-halogen bonds in these clusters appear to be less susceptible to *in vivo* cleavage than carbon-halogen bonds [22-25].

Drug design involving boron-containing agents has two major disadvantages compared with conventional drug design: 1) There is a lack of compound libraries containing boron agents for biological high-through put- and/or virtual screening [26], and 2) many software packages available for structure/ligand-based drug design do not have inbuilt parameters for boron atoms. Several strategies to circumvent the latter problem have been reported in recent years. These include the substitution boron with carbon [27-30,16, 15,8,31,32] and the calculation of suitable boron parameter for specific applications [33]. Similar methods have been used to obtain physicochemical parameters of boron compounds [13,34,35]. Other reports dealing with docking studies of boron compounds do not provide specific information on computational strategies addressing this problem [19,36].

Crystal structures of proteins complexed with carboranyl drugs have been reported recently [20,21]. These provide for the first time the opportunity to verify docking strategies that have been developed previously [28,29] for carboranyl compounds. In the present paper, we describe the docking of *closo*- and *nido*-carboranyl antifolates into the active site of a human dihydrofolate reductase (hDHFR) using Autodock, and we compare the obtained docking poses with the poses of the same antifolates in the original hDHFR-carboranyl antifolate crystal structure [20].

Methods

Hardware/Software. Sybyl 7.1 (Tripos Inc., St Louis MO) installed on a Silicon graphics O2 workstation. HyperChem 7.51 (Hypercube Inc., Gainsville, FA), PyMoL(DeLano Scientific LLC., San Francisco, CA), and Gaussian 03W(Gaussian Inc. Pittsburgh, PA) installed on a Dell Optiplex GX270 desktop computer. AutoDock Tools (ADT), Autogrid4, and AutoDock4 (Molecular Graphics Laboratory, The Scripps Research Institute, La Jolla, CA), installed on Red Hat Enterprise, Linux 5 desktop computer. Maestro 8 (Schrodinger Inc., Portland OR) installed on an Opensuse 10.1

desktop computer. Molecular optimization and charge calculations with Gaussian as well as all docking jobs were performed with an AMD opteron processor-team HPC cluster.

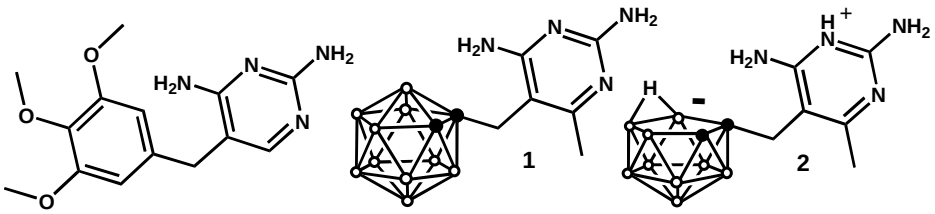

Figure 2: Trimethoprim, *closo*-carboranyl antifolate (**1**), and *nido*-carboranyl antifolates (**2**).

Crystal Structures. Dimeric human dihydrofolate reductase (hDHFR) with [5-(1,2-*closo*-dicarbadodecarboran-1-yl)methyl]-2,4-diamino-6-methyl-pyrimidine (**1**) (PDB ID # 2C2S) or a racemic mixture of [5-(7, 8-*nido*-dicarbaundecarboran-7-yl) methyl]- 2,4-diamino-6-methylpyrimidine (**2**) (PDB ID # 2C2T) (**Fig. 2**) [20].

Ligand Construction and Optimization. Closo-carboranyl antifolate **1** and both enantiomers of *nido*-carboranyl antifolate **2** were extracted from the hDHFR crystal structures. Hydrogen atoms were added and Mulliken-, APT-[42] and ESPfit charges were calculated with Gaussian at AM1 (37,38) and HF/6-31+G* level (**Table 1**) [39-41]. Ligands **1** and **2** were also prepared with HyperChem without using any crystallographic coordinates (referred to as "constructed" henceforth). Optimization and partial atom charge calculations of constructed **1** and **2** were carried out with Gaussian at HF/6-31+G* level (**Table 1**). For both extracted and constructed forms of **2**, *closo*- and *nido*-carborane moieties were aligned and the coordinates for the appropriate boron atom of the *closo*-structure were used to insert the extra-hydrogen into the *nido*-structures. Original small molecule crystal structure data for uncomplexed **1** and **2** were generously provided by Dr. Steven Ealick, Cornell University, Ithaca, NY, and Dr. David Borhani, Harvard Medical School, Boston, MA.(20) Mulliken charges were calculated at HF/6-31+G* level with Gaussian for these small molecule crystal structures before docking (**Table 1**).

Docking. The protein structures were prepared using the structure preparation tool of Sybyl. Monomers were separated from both crystal structure and the blocking groups AMI and CXC, respectively, were added to the N- and C-terminis for neutralization. Water molecules were removed,

hydrogen atoms were added, and side chain amides and side chains bumps were fixed. NADPH remained in the structures. Constructed forms of **1** and **2** were aligned with their extracted counterparts using Sybyl or Maestro before docking. Uncharged protein structures were imported from Sybyl into ADT. AutoDock atom types were assigned and the Gasteiger charges were added. Autodock has no default parameters for boron atoms and no dummy atom option. Therefore, boron atom types were changed to 'C' atom types after converting the mol2 file into pdbqt format with ADT. It was also made sure that all the non polar hydrogens were merged before saving the file into pdbqt format. Bonds within the carborane cages that were recognized as rotatable by AutoTors in ADT were changed to non-rotatable bonds. The TORSDOF for the written output files of all ligands was set to 2. The *Lamarckian genetic algorithm* (LGA) was selected for ligand conformational search. The docking area was defined using Autogrid. 40x40x40 3-D affinity grids centered around the antifolate binding site with 0.375 A˚ spacing were calculated for each of the following atom types: (a) protein: A (aromatic C), C, HD, N, NA, OA, SA; (b) ligand: C, A, HD, N, NA, e (electrostatic), and d (desolvation). Additional docking parameters were: Dockings trials: 100, Population size: 250, random starting position and conformation, translation step ranges: 2.0 Å, rotation step ranges: 50, elitism: 1, mutation rate: 0.02, crossover rate: 0.8, local search rate: 0.06, and energy evaluations: 25,000,000. Final docked conformations were clustered using a tolerance of 2Å root-mean-square deviations (RMSD).

Results and Discussion

*Binding characteristics of the closo- and nido-cages of the carboranyl antifolates **1** and **2** in the active site of hDHFR.* The boron-bound hydrogen atoms (1-6, 9-11) in carboranes may have negative partial charges.(43,44) According to ESPfit calculations, the average partial charges for these hydrogens are about -0.24 for two-fold negatively charged *nido*-carborane $[C_2B_9H_{11}]^{2-}$, -0.15 for one-fold negatively charged *nido*-carborane $[C_2B_9H_{12}]^{-}$, and -0.07 for neutral *nido*-carborane $[C_2B_9H_{13}]$. These charges are consistent with those generated in our own ESPfit calculations for **1** and **2**. However, Mullikan charges, obtained at

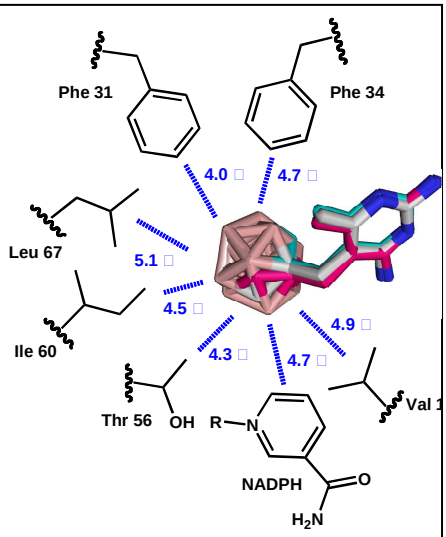

Figure 3: Hydrophobic binding of **1** (gray) and both enantiomers of **2** (cyan, magenta) in the active site of hDHFR. Coordinates for **1** and **2** were extracted from the crystal structures of hDHFR. All structures were overlaid. Distances are indicated for **1**.

HF/6-31+G* level for **1** and **2**, were generally more positive and produced similar docking accuracy (**Table 1**). It has been suggested that the hydrogens in the aforementioned *nido*-carboranes form proton–hydride type bonds rather than conventional hydrogen bonds with nitrogen-bound hydrogen atoms in tetrapeptides [43].

Closo-carboranyl antifolate **1** and both of its enantiomeric *nido*-carboranyl counterparts (**2**) showed almost identical binding patterns in the hDHFR crystal structures, as indicated in **Fig. 3** [20]. The additional BH vertex of the *closo*-cage of **1** is positioned slightly above the center of the open faces of the *nido*-cages of **2**. This binding pattern is similar to that of trimethoprim in the active site of hDHFR [20]. The clusters of **1** and **2** bind in the same hydrophobic pocket of hDHFR as the trimethoxyphenyl group of trimethoprim [20]. As shown in **Fig. 3**, the binding between amino acid residues of this pocket with both the neutral *closo*-cage of **1** and the presumably negatively charged *nido*-cages of **2** are typical for hydrophobic interactions. Proton–hydride type bonds do not seem to play any role in these interactions, as is evident from the orientation of the hydroxyl group in Thr56, which points away from the cage. Similar hydrophobic interactions were observed for a metallocarborane, consisting of two negatively charged *nido*-carboranes, bound to HIV protease [21]. In a recent study with *closo*- and *nido*-carboranyl nucleosides [45], those containing neutral *closo*-carboranes demonstrated their highly hydrophobic character by forming aggregates in aqueous solution. Negatively-charged *nido*-carboranyl nucleosides, however, did not aggregate.

Table 1: Optimization characteristics and docking patterns of antifolates.

Closo-carboranyl Antifolate (**1**)						
	AM1		HF/6-31+G*			
	Mulliken (extract.)	APT (extract.)	Mulliken (extract.)	Mulliken (construct.)	ESPfit (extract.)	Mulliken (SMCS[g])
BH[a]	0.079	-0.141	0.118	0.110	-0.0491	0.116
CH[b]	0.162	0.117	0.345	0.326	0.181	0.321
# of Poses[c]	100	99	100	84	100	100
MBE[d]	-9.45	-4.58	-3.93	-1.60	-5.09	-3.28
RMSD[e]	0.61-0.62	0.68-0.72	0.61-0.62	0.67-0.84	0.57-0.61	0.69-0.71
Nido-carboranyl Antifolate **(2)**[f]						
BH[a]	0.028	-0.099	0.078	0.110	-0.106	0.042
CH[b]	0.101	0.033	0.247	0.234	0.096	0.239
# of Poses[c]	100	100	100	45	100	100
MBE[d]	-8.13	-4.47	-2.19	-1.96	-2.37	-2.80
RMSD[e]	0.64-0.65	0.64-0.65	0.71-0.80	0.47-0.60	0.63-0.65	0.65-0.73

[a]Average partial charge of all cage hydrogen atoms connected to boron; [b]partial charge of cage hydrogen atom bound to carbon; [c]# of docked poses of antifolate within a cluster with lowest RMSD (out of 100) ; [d]Mean Binding Energy, [e]RMSD (Å) range for docked antifolate poses within the selected cluster, [f]enantiomer designated as 2B in [20]; [g]Small Molecule Crystal Structure parameter [20].

Docking studies. An analysis of the docking studies with **1** and **2** is shown in **Table 1** and in **Fig. 4**. It is apparent that minor differences in geometries (as a result of different data sources and/or optimization methods/calculations), MBEs, and partial atom charges did neither impact the docking accuracy of *closo*-carboranyl antifolate **1** nor that of *nido*-carboranyl antifolate **2** to a significant extent. Both enantiomers of **2** showed similar docking patterns (data not shown). In particular the 2,4-diamino-6-methylpyrimidine portions of docked **1** and **2** showed the same placements and orientations as those of their counterparts in the original protein-ligand crystal structure, while carborane cluster placements and orientations revealed minor differences (**Fig. 4**).

AutoDock4 is based on a *Lamarckian genetic algorithm* (LGA) method [46,47]. Basically, this program determines total interaction energies between random pairs of ligands and various selected portions of protein to determine docking poses. The docking algorithm in FlexX,(48) another popular docking program, is based on an incremental construction method of ligands in the protein. FlexX docking of **1** and **2** into hDHFR crystal structures resulted in increased RMSD values as compared with Autodock docking (data not shown).

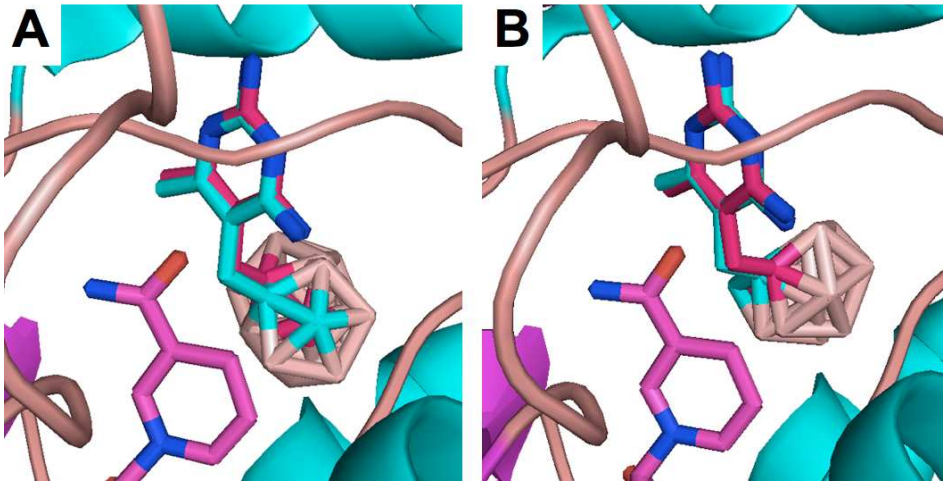

Figure 4: (A) Overlap of the docked pose of constructed **1** (cyan) with the corresponding pose of **1** (magenta) in the original crystal structure (RMSD range: 0.67-0.84). (B) Overlap of the docked pose of constructed **2** (cyan) with the corresponding pose of **2** (magenta) in the original crystal structure (RMSD range: 0.47-0.60).

Conclusion

The binding of the *closo*-carborane moiety of **1** and the *nido*-carborane moiety of **2** into a hydrophobic pocket of the hDHFR crystal structure was mediated through van der Waals interactions only. Proton–hydride type bonds did not seem to contribute to the binding. Docking of **1** and **2** was accurate and

robust. It was not influenced significantly by minor variations in geometries and partial atom charges. Botta et al. [33] discussed the need for boron parameter implementation in computational studies with compounds containing single tetrahedral boronate function to appropriately simulate conformational flexibility. However, boron clusters are rigid and highly hydrophobic. Therefore, conformational flexibility may not play an important role in docking studies with carboranyl species. The observed docking patterns of *closo*- and *nido*-carboranyl antifolates with hDHFR validate previously developed methods for structure and ligand-based design of carborane-containing compounds [27-30,15,16,8, 31,32], which are based on the simple replacement of boron atoms with carbon atoms within the cage structures.

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CARBORANES AND METALLACARBORANES AS BUILDING BLOCKS FOR BIOMATERIALS

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Summary

The great chemical and physical properties of Polypyrrole membranes doped with $[Co(C_2B_9H_{11})_2]^-$ have been applied in the development of novel hydrogen, potassium and sodium ion selective electrodes (IES's). To apply in biomedical measurements, these membranes have been used as the solid internal contact in potentiometric microelectrodes by using microfabrication technologies and electrochemical polymerization. It is foreseen that insoluble amine salts of $[Co(C_2B_9H_{11})_2]^-$ can be used in potentiometric PVC based ISE's for the detection of antibiotics. Additionally Boron iodinated *closo o*-carborane derivatives have been synthesized for possible medical applications in radioimaging/radiotherapy and as X- Ray contrast agents. In addition, high Boron water soluble *o*-carborane derivatives have been synthesized to be used as anti-tumor agents for BNCT.

Key Words: Ion Selective Electrodes, Conducting Organic Polymer, Metallacarboranes, regioselectivity.

Introduction.

Boron clusters, boranes and carboranes, display many particular characteristics that do not find parallel in their organic counterparts. These cage like molecules composed of boron and hydrogen atoms, hold great promise for use in the treatment of nuclear waste [1], cancer treatment [2] and boron neutron capture therapy. To be used in biological systems, they need to be functionalised [3] on the boron cage, and be made soluble in water. The cluster C-H vertices may be deprotonated with strong bases, and later substituted [3]. Conversely, boron-substituted carboranes are less developed because of the higher difficulty of introducing functional groups at the boron atoms of the carborane cage.

It has been shown that microelectrodes are valuable to measure impedance, hydrogen and potassium concentrations in organ tissues, provide relevant information during cardiac surgery [4] or in the evaluation of organ transplantation viability. The development of microfabricated ion-selective sensors for detecting electrolytes such as K^+ and Na^+, as well as pH, in both blood [5] and in cardiovascular research and monitoring [6] is a growing

interesting area. Copper is an essential trace element for most animals. It is fundamental for the absorption and use of iron to make haemoglobin, and is part of several enzymes that have a fundamental role in preventing oxidation, manufacturing collagen and producing energy [7]. For these reasons, a number of ion selective electrodes (ISE) based on various carrier types have been developed.

The view of boranes or carboranes as rare, strange or esoteric, in addition to have high price and an assumed, unrealistically, low stability precludes synthetic chemists in other areas to think on these clusters as real building blocks. The advent of materials science with emphasis in new tridimensional structures should correct this vision. In this workshop we report briefly on some recent results of possible medical applications developed in our group in which the carborane or metallacarborane species play a crucial role.

Metallacarboranes in Analytical Medical Chemistry

Metallacarborane anions as doping agents in conducting organic polymers (COP) and their use as Ion-selective electrodes (ISE).

The synthesis of PPy by electropolymerization of the monomer in the presence of the weakly coordinating anion $Cs[Co(C_2B_9H_{11})_2]$, produces a polymer in which the anion is an integral part of the material [8]. The retainment of the doping anion in the polymeric structure, and its uniform distribution throughout the film has been established by an in-depth analysis with argon-ion sputtering, demonstrating for the first time uniform distribution of the doping agent through the whole thickness of the material. The confinement of the cobaltabis-carbollide is in contrast with more mobile simple doping counter ions that are easily de-inserted upon reduction. The reclusion of $[Co(C_2B_9H_{11})_2]^-$ in the structure must be due to its large size and hydrophobicity. The electrochemical cycling of these PPy materials involves the insertion and de-insertion of cations during reduction and oxidation, respectively. It has also been demonstrated that the incorporation of $[Co(C_2B_9H_{11})_2]^-$ in the PPy material has dramatically improved its overoxidation threshold, as compared with commonly used doping anions (Fig. 1). The high volume of $[Co(C_2B_9H_{11})_2]^-$ imposes low mobility inside the polymeric matrix thus preventing dopant leakage when a reducing potential is applied to the material.

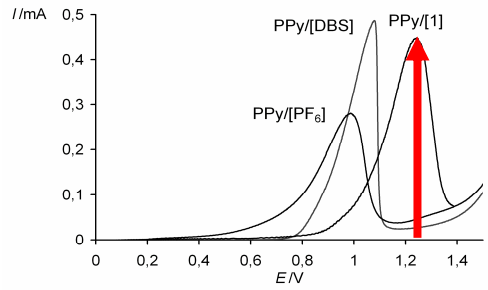


Figure 1. Overoxidation resistance limit of the PPy doped with $[Co(C_2B_9H_{11})_2]^-$.

Under these conditions the cation capture prevails during the reversible electrochemical redox process, also called doping/undoping.

$$[PPy^{n+}(A^-)_n] + nC^+ + n\,e^- \leftrightarrows [Ppy(A^-)_n(C^+)_n] \text{ (Equation 1)}$$

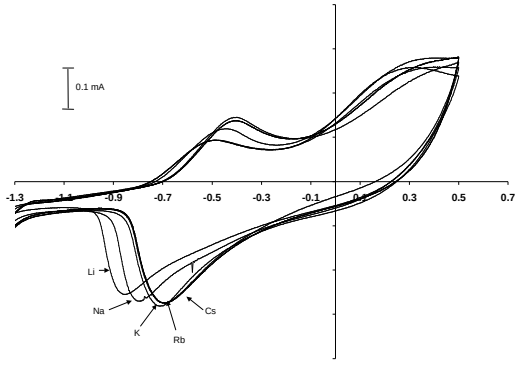
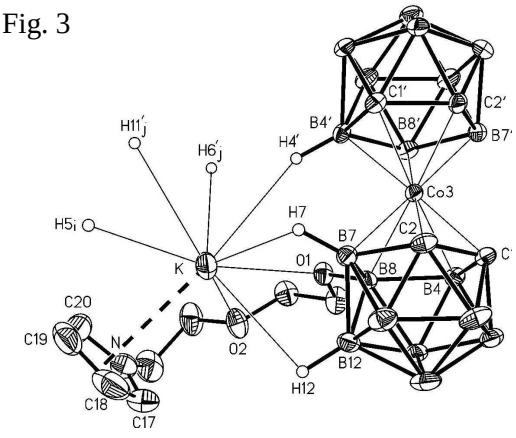


Figure 2: Cyclic voltammetry of PPy doped with $[Co(C_2B_9H_{11})_2]$ in aq. Alk. Cl.

The membrane is highly sensitive to the cationic volume of the solute. This property has allowed to develop cationic selective membranes (H^+, Li^+, Na^+, K^+, Rb^+, Cs^+) [9] "intelligent membranes" by control of the applied reducing potential (Fig. 2) and can be used as a simple PVC-PPy electrode for pH measurements and titration. The chronocoulometries registered during the charge-discharge process show an almost perfect reversibility of cation exchange process and no detectable degradation of the membrane due to dopant loss or overoxidation even after 40 successive cycles. Some physical properties of the polymer must be attributed to the unique nature of these low-density, low-coordinating, low-nucleophilic anions, but some may be attributed to the large boron presence in the material. The preparation of self doping conducting polymers has been obtained with sulfonate, phosphonate, benzoate, and carboxilate bearing side chains [10]. The self-doping anions have the additional advantage over non-chemically bonded anions that are not exposed to leakage in the oxidation/reduction process [11]. Formally this can also be achieved with high molecular-weight anions due to a severe reduction of the anionic movement within the polymer [12]. In the aim of exploring and further improving the possibilities of the low charge density anion $[Co(C_2B_9H_{11})_2]^-$ we have to covalently bound the $[Co(1,2\text{-}C_2B_9H_{11})_2]^-$ anion to a pyrrole unit via an spacer to get a self-doped material. Two strategies have been possible: to bind the pyrrolyl bearing fragment to the cluster carbon, or to do it on the boron. The

Fig. 3



first proved to be impractical as only a 2% yield of the precursor was obtained [13]. The second has been possible through the development of the chemistry of $[Co(8\text{-}C_4H_8O_2\text{-}1,2\text{-}C_2B_9H_{10})(1',2'\text{-}C_2B_9H_{11})]$ [14]. The reaction of this neutral species having a positive oxygen on the boron with potassium pyrrolyl has provided the potassium salt of the target molecule $[Co(8\text{-}C_4H_4N\text{-}C_4H_8O_2\text{-}1,2\text{-}C_2B_9H_{10})(1',2'\text{-}C_2B_9H_{11})]^-$ incorporating a pyrrole unit covalently bonded to the $[Co(C_2B_9H_{11})_2]^-$ unit by a diether aliphatic chain (Fig. 3) [15].

As the pyrrolyl derivatization is performed on the nitrogen atom, the α carbon atoms remain unsubstituted ready for subsequent polymerization of the monomer. The direct polymerization of the monomer as well as copolymerization with pyrrole units leads to the formation of a new group of self-doped conducting polymer,[i] even improving the outstanding properties described for PPy-$[Co(C_2B_9H_{11})_2]$ [8]. As the doping agent is inherently bonded to the pyrrole unit no supporting electrolyte is necessary during polymerization. The copolymer really improves the already high overoxidation resistance measured for non-covalently linked PPy$[Co(1,2\text{-}C_2B_9H_{11})_2]$ (current peak at 1,25V) and display an extraordinary overoxidation resistance that shall widen the possibilities of these materials. We observed that the conducting organic polymer adequately doped with $[Co(C_2B_9H_{11})_2]^-$ grafted to the PPy strands through ether and alkane spacers spontaneously self-repairs in few hours after having been taken beyond the formal overoxidation potential and consequently after having been dead [17]. A new Phoenix Bird arises out of the ashes to live again (Fig. 4). The resulting membrane acts as a cation exchanger with the media and therefore is an excellent candidate for the implementation of selective ion exchange resins controlled by an electrochemical potential.

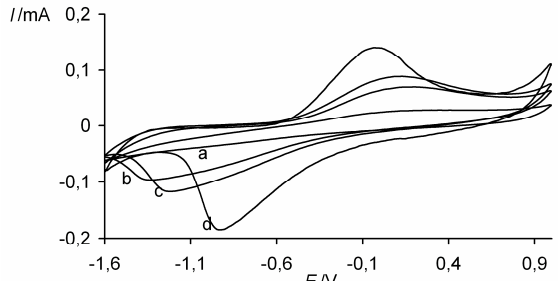


Figure 4. Gradual activity recovery of self doped PPy. a) immediately after the insult at 1300 mV, b) after 30 minutes, c) after 1 h and d) after 18h.

All-solid-state hydrogen sensing <u>microelectrodes</u> based on novel PPy$[Co(C_2B_9H_{11})_2]$ as a solid internal contact.

Ion-selective electrodes (ISE) are commonly used for the determination of a wide range of analytes but biomedical measurements require the use of miniaturized ISE's. The traditional configuration of ISE's with an internal filling solution is not suitable for miniaturization and this has led to the development of all solid-contact ion selective microelectrodes (SCISMEs). The physical and chemical properties of the PPy doped with $[Co(C_2B_9H_{11})_2]^-$ have

been applied in the development of novel hydrogen, potassium and sodium in ion selective potentiometric microelectrodes using microfabrication [18] technologies and electrochemical polymerization. The potentiometric microelectrodes with PPy[$Co(C_2B_9H_{11})_2$] as the solid internal contact have been fabricated by immobilization of the corresponding ionophores on the surface of platinum microelectrodes modified by the conducting PPy[$Co(C_2B_9H_{11})_2$] polymer.

The schematic structure of the SCISMEs microelectrodes cross section in silicon needle-shaped substrates is shown in Fig. 5 [18]. Four platinum microelectrodes are fabricated in each silicon needle. The dimensions of an individual electrode are 300μm×300 μm. Images of one of these microelectrodes at the different steps of the preparation process are shown in Figure 5: (b) platinum microelectrode, (c) microelectrode with the conducting PPy layer and (d) microelectrode with the PVC based membrane containing the ionophore.

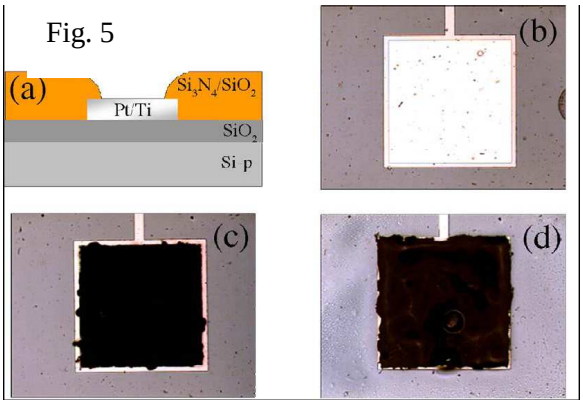


Fig. 5

The ion selective membranes, that are based on a plasticized poly(vinyl chloride) (PVC) membrane, contain the ionophore and are deposited on top of a layer of the conducting PPy[$Co(C_2B_9H_{11})_2$]. The ionophores are: tri-*n*-dodecylamine for H^+, 1,3-(di-4-oxabutanol)-calix[4]arene-crown-5 for K^+ and *p-tert*-butylcalix[4]areneethylester for Na^+.

H^+ microelectrode: It exhibited excellent selectivity for the primary ion and a linear response over the pH range 3.5–11.0 with a slope of 52±2 mV $decade^{-1}$. The selectivity versus Li^+, Na^+ and K^+ was examined and the results obtained were comparable to these of a conventional ion-selective electrode (ISE) based on the same ionophore. Response time was also investigated, and the time required to achieve 90% of steady state potential, when the pH was decreased from 7.12 to 6.87, was less than 45 s.

K^+ microelectrode: The response of the microelectrode was linear with a Nernstian slope of 51±2mV per decade over a K^+ ion concentration range of 6×10^{-6} to 1×10^{-1} M, with a detection limit of 1.8×10^{-6} M. The microelectrode is suitable for use within the pH range of 3–11 and could be used for at least one month without a considerable alteration in its potential.

Na^+ microelectrode: The microelectrode shows a linear response for Na^+ concentrations between 3.0×10^{-6} and 1.0×10^{-1}M with a Nernstian slope of

58.65±2mV per decade and a detection limit of 1.45×10^{-6} M. The response time was 14 s, and the electrode is suitable for use within the pH range of 3–10.

The outstanding selectivity of these all-solid-state ion-sensitive microelectrodes makes them attractive for biological and physiological applications. They are well suited for the detection of myocardial ischemia during cardiac surgery.

Carboranes and Metallacarboranes as drugs in Medicine.

Synthesis of Boron iodinated closo o-carborane derivatives.

While perfluorination [19] and perchlorination [20] of dicarbaboranes has been achieved, perbromination and periodination of *o*-carborane had never been successful. The electrophilic monoiodination at the 9 position [21] and diiodination at the 9 and 12 vertices [21a,22] of the *o*-carborane cage was achieved by treatment with I_2 in the presence of $AlCl_3$ [22,23]. A highly iodinated I_8-1,2-*closo*-$C_2B_{10}H_4$ was later obtained by reaction with ICl and triflic acid [24]. The combination of nucleophilic and electrophilic processes allow the regioselective synthesis "on demand" of several B-iodinated derivatives of *o*-carborane and their periodination (Fig. 6) [25]. Boron substitution on boron1,2-*closo*-$C_2B_{10}H_{12}$ cluster could be obtained with the Pd-catalysed conversion of B-I to B-C with Grignard reagents, RMgBr (R = alkyl or aryl group). Therefore, regioselective Boron substitution relies on Boron iodination. To this aim, not only conventional reactions on solution have been studied, but also a highly efficient, clean and fast solvent free procedure [26] has been studied that has provided successful results to regioselectively produce B-iodinated *o*-carborane derivatives with a careful control of the reaction conditions. The acidity of the hydrogen atoms bonded to $C_{cluster}$ is strongly dependent upon the degree and type of B-substitution. Iodination accentuates the acidity while methylation decreases it.

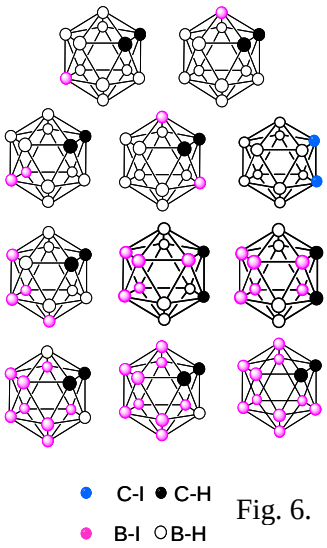


Fig. 6.

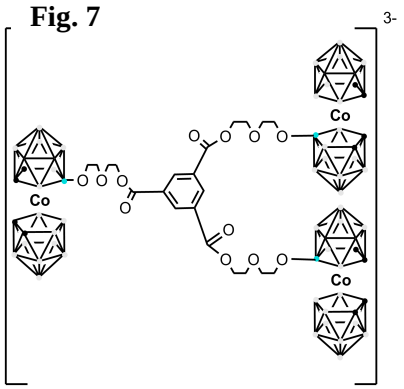
We proved [27] that low Boron iodinated *closo*-*o*-carborane derivatives could undergo catalyzed isotopic exchange between the natural iodide of iodinated carboranes and [^{125}I] iodide to provide [^{125}I]-labeled carboranes. The reaction was carried out on 3-I-1,2-*closo*-$C_2B_{10}H_{11}$ and 9-I-1,2-*closo*-$C_2B_{10}H_{11}$ by using Herrmann's catalyst in toluene at 100 ºC under argon. Exchange of natural iodine by [^{125}I] took place in 98.1 and 98.6 % yield for 3-I-1,2-*closo*-$C_2B_{10}H_{11}$ and 9-I-1,2-*closo*-$C_2B_{10}H_{11}$ respectively in only 5

min. Low Boron iodinated *closo-o*-carborane derivatives with halogens could be useful as radioimaging/radiotherapy for biomedical applications such as in pharmacokinetic studies of boron compounds for boron neutron capture therapy. Since the conventional X-Ray contrast organic media have from 30-70% iodine (w/w), high iodine-containing *closo o*-carborane derivatives (I_8-1,2-*closo*-$C_2B_{10}H_4$, 88.1% iodine; I_9-1,2-*closo*-$C_2B_{10}H_3$, 89.4% iodine, and I_{10}-1,2-*closo*-$C_2B_{10}H_2$, 90.45% iodine) could be useful as X- Ray contrast agents for selective visualization of organs or structures. The contrast agent amount that must be administrated to produce opacity will be less the higher its contents in iodine is so to avoid the consequent risks of unwanted effects.

Synthesis of high Boron o-carborane derivatives to be used as anti-tumor agents for BNCT(Boron Neutron Capture Therapy).

A set of "Fréchet type" dendrons have been functionalized with different neutral and anionic carborane clusters. One of the main characteristics of these compounds is their photo-luminescent properties due to the presence of the cluster. Additionally the anionic compounds are soluble in water that can be important for medical applications. We have also prepared a family of first and second generation of neutral dendrimers containing four or eight *closo*-carborane clusters on the periphery using both divergent and convergent approaches. The modification of the *closo*-cluster has been carried out by degradation reaction using KOH/EtOH, to obtain the corresponding polyanionic carbosilane dendrimers containing peripheral *nido*-carborane clusters [28].

Fig. 7



The zwitterionic compound [3,3'-Co(8-$C_4H_8O_2$-1,2-$C_2B_9H_{10}$)(1',2'-$C_2B_9H_{11}$)] was reported for the first time by the reaction of the parent $Cs[Co(C_2B_9H_{11})_2]$ metallacarborane with H_2SO_4–Me_2SO_4 in 1,4-dioxane in 1996 [29]. Later, the same compound was obtained in a higher yield (94 compared to 45 %) and a better work-up procedure by using $BF_3 \cdot OEt_2$ [30]. The related tetrahydropyrane-based derivative [3,3' Co(8-C_4H_8O-1,2-$C_2B_9H_{10}$)(1',2'-$C_2B_9H_{11}$)] was also prepared [15]. The zwitterionic compound [3,3'-Co(8-$C_4H_8O_2$-1,2-$C_2B_9H_{10}$)(1',2'-$C_2B_9H_{11}$)] has been shown to be susceptible to nucleophilic attack on the positively charged oxygen atom, e.g. by pyrrolyl [15], imide, cyanide or amines [31], phenolate, dialkyl or diarylphosphite [32], N alkylcarbamoyldiphenylphosphine oxides [33], alkoxides [30,34] and

nucleosides [35] resulting in one anionic species formed by the opening of the dioxane ring. A recent review [36] updates the synthesis of different oxonium derivatives of polyhedral boron hydrides. The ring-opening reactions of cyclic oxonium derivatives of polyhedral boron hydrides with sulfur nucleophiles are rare and include the ring-opening reactions of cyclic oxonium derivatives of the *closo*-dodecaborate and *closo*-decaborate anions with hydrosulfide [37]. The reaction of the tetramethylene oxonium derivative of the *closo*-dodecaborate anion with lithium derivatives of *o*-carborane gives the $[C_2B_{10}]$-$[B_{12}]$ double-cage boron compounds [38].

We have just reported [39] the synthesis of multianionic species (Fig. 7) by using Grignard reagents, carboxylic acid, carboranes and thiocarboranes as nucleophiles. This feature makes them potential systems to be use as "drug delivery" or inhibitors of viral infection.

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ASYMMETRIC SYNTHESIS OF 5-LIPOXYGENASE INHIBITORS

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Summary

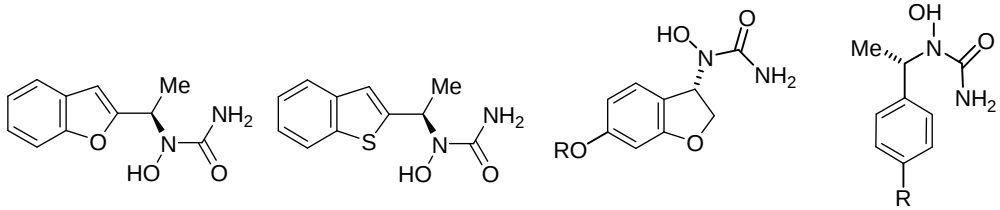
The asymmetric synthesis of N-substituted *N*-hydroxyureas, derived from acetophenones, 1-(benzofuran-2-yl)ethanone, 2,3-dihydrobenzofuran-3-one, 1-(benzo[*b*]thiophen-2-yl)ethanone, is described. The enantioselective reduction of these ketones and /or their oxime ethers with borane /oxazaborolidines was used as the key step generating the stereogenic center linked to the *N*-hydroxyurea moiety.

Key words: borane/oxazaborolidines, N-substituted *N*-hydroxyureas.

Introduction

5-Lipoxygenase is involved in the first step of the biosynthesis of leukotrienes, mediators in several allergic and inflammatory diseases, such as asthma, inflammatory bowel disease, psoriasis, and other [1-3]. A search for inhibitors of 5-lipoxygenase in the last two decades resulted in the discovery of N-substituted *N*-hydroxyureas. Various compounds of this class were prepared and *N*-1-(benzo[*b*]thiophen-2-yl)ethyl-*N*-hydroxyurea (Zileuton®) was introduced on the market in USA as an anti-asthmatic drug [4-5]. Later, other *N*-hydroxyureas have been prepared and at present are in various stages of clinical tests [6-8]. Zileuton and similar 5-lipoxygenase inhibitors have the *N*-hydroxyurea moiety located at a stereogenic center, and their enantiomers exhibit different inhibiting activities. Consequently, asymmetric synthesis is desired, although in certain cases racemates can be used. Three 8–12 steps asymmetric syntheses of Zileuton, using a chiral pool precursor or chiral auxiliaries, have been developed [9-11]. Similarly, a chiral auxiliary was used in the asymmetric synthesis of *N*-(2,3-dihydrobenzofuran-3-yl)-*N*-hydroxyurea [6].

In this study on the asymmetric synthesis of *N*-hydroxyureas **1**–**7**, we focused on the enantioselective reduction of ketones and oxime ethers, as a key step generating the stereogenic center linked to the *N*-hydroxyurea moiety.



1

2
Zileuton®
(Abbott Laboratories)

3, R = Me
4, R = Bn

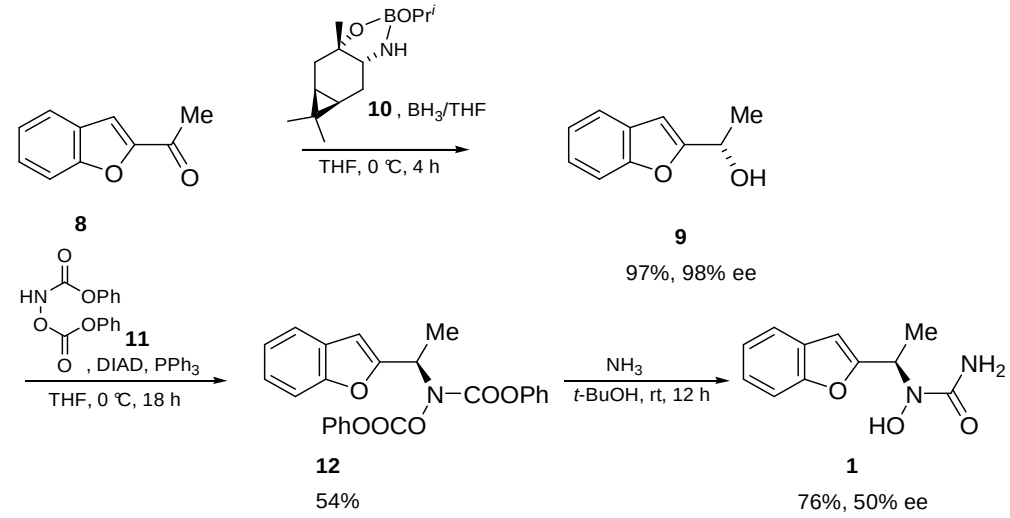
5, R = H
6, R = MeO
7, R = BnO

Results and discussion

Based on our earlier studies on the enantioselective reduction of prochiral ketones and oxime ethers [12,13], and literature reports in this area [14-17], borane/oxazaborolidines was selected as the preferred reducing agent generating the stereogenic center.

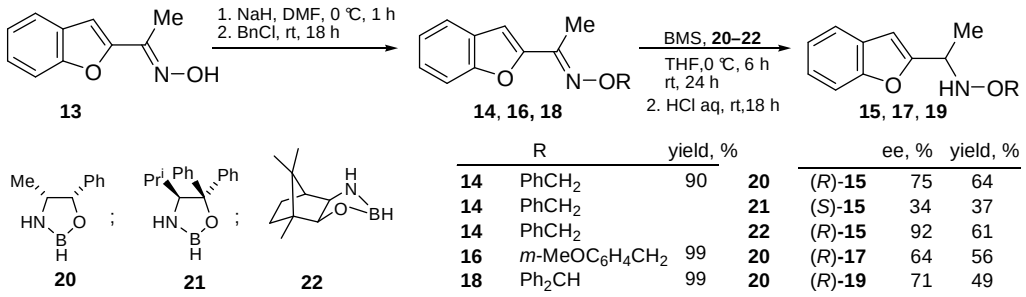
In the first approach to (*R*)-*N*-1-(benzofuran-2-yl)ethyl-1-hydroxyurea **1**, 1-(benzofuran-2-yl)ethanone **8**, readily prepared from salicylic aldehyde and chloroacetone [18], was reduced with borane in the presence of oxazaborolidines generated from various β-amino alcohols, and the highest enantiomeric excess of the product alcohol **9**, 98% ee, was obtained when oxazaborolidine **10**, prepared from triisopropoxyborane and (1*S*,3*S*,4*R*,6*R*)-4-amino-3,7,7-trimethylbicyclo[4.1.0]heptan-3-ol [19], was used. The Mitsunobu reaction of alcohols is a highly stereospecific substitution of the hydroxyl group proceeding with inversion of configuration. The use of nitrogen nucleophiles in this reaction provides a convenient access to nitrogen derivatives, and *N*,*O*-bis(diphenoxycarbonyl)hydroxylamine **11** seemed a suitable nucleophile for our synthesis. The product of its reaction with alcohols under Mitsunobu conditions is readily transformed into the corresponding *N*-hydroxyurea by treatment with ammonia. Consequently, this sequence: the reduction of ketone – the reaction of the product alcohol with **11** – treatment with ammonia, provides a short access to *N*-hydroxyureas. Unfortunately, application of the sequence to **9** resulted in partial racemization leading to **1** of 50% ee, obtained in 76% yield [20]. (Scheme 1).

Apparently, the electron donating effect of the electron-rich benzofuran-2-yl group makes the reaction center prone to racemization. Alcohols containing such groups, e. g., secondary *ortho*- and *para*-alkoxybenzylic alcohols, also undergo partial racemization under Mitsunobu conditions [21,22]. In the second approach, **8** was converted into oxime **13** and its *O*-benzyl ether **14** was reduced with borane/oxazaborolidine, generated from (1*S*,2*R*)-norephedrine, producing (*R*)-1-(benzofuran-2-yl)ethylamine, 75% ee (Scheme 2).



Scheme 1

Searching for higher enantioselectivity, *m*-methoxybenzyl ether **16** and benzhydryl ether **18** were reduced with the same reagent, however, hydroxylamine ethers (*R*)-**17** and (*R*)-**19** of lower enantiomeric excess were obtained. The reduction of **14** with borane/oxazaborolidine, generated from (*S*)-diphenylvalinol, was much less selective producing (*S*)-**15** of only 34% ee. Then, we turned to terpene oxazaborolidines selecting (1*R*,2*S*,3*R*,4*S*)-3-amino-1,7,7-trimethylbicyclo[2.2.1]heptan-2-ol **26** (Scheme 3) for the generation of the corresponding oxazaborolidine **22** (Scheme 2). The reduction of **14** with borane/**22**, produced (*R*)-**15**, 92% ee, in 61% yield. Noteworthy are two aspects of the reaction. First, much higher enantioselectivity of the reduction in the presence of this terpenic oxazaborolidine, as compared to the above mentioned oxazaborolidines. Second, the possibility of halting the reaction at the first reduction step producing hydroxylamine ether.

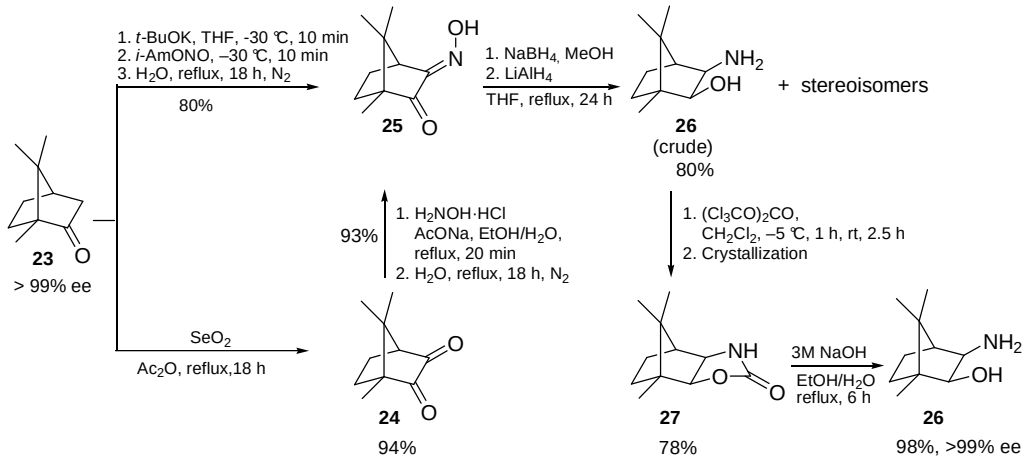


	R	yield, %		ee, %	yield, %
14	$PhCH_2$	90	**20**	(*R*)-**15**	75	64
14	$PhCH_2$		**21**	(*S*)-**15**	34	37
14	$PhCH_2$		**22**	(*R*)-**15**	92	61
16	*m*-$MeOC_6H_4CH_2$	99	**20**	(*R*)-**17**	64	56
18	Ph_2CH	99	**20**	(*R*)-**19**	71	49

Scheme 2

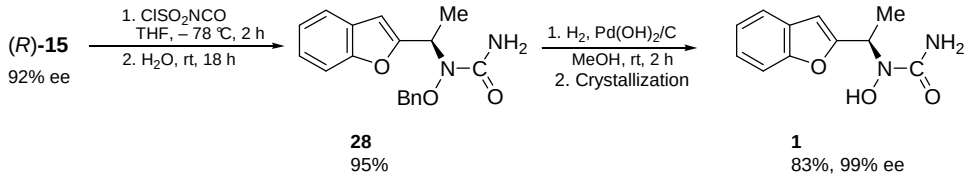
β-Amino alcohol **26** has been described several times in the literature, and can be prepared from readily available optically pure (1*R*)-camphor in two steps. However, when we undertook the synthesis, it turned that the preparation of pure **26** requires an efficient purification procedure. The synthesis is presented on Scheme 3.

(1*R*)-Camphor **23** can be readily transformed into (1*R*)-3-isonitrosocamphor **25,** either by oxidation with selenium dioxide to (1*R*)-camphoroquinone **24** and its monooximation, or by treatment with a strong base followed by nitrosation with isoamyl nitrite. (1*R*)-3-Isonitrosocamphor **25** is obtained as an *E/Z* mixture, the ratio depending on the base and the reaction conditions. The *E/Z* ratio affects the stereochemical outcome of the next reduction step. Fortunately, it can be readily changed to 97:3 by heating the mixture with refluxing water. The stepwise reduction of **25**, first the keto group with sodium tetrahydroborate, and then the oxime group with lithium tetrahydridoaluminate, produces **26**. The same product is obtained by direct reduction of **25** with lithium tetrahydridoaluminate. Varying amounts of the *endo,endo* stereoisomer **27** and other stereoisomers are also formed, depending on the reaction conditions. Several purification attempts by crystallization of crude **26** failed. Purification by treatment with methanesulfonic acid, succinic acid, tartaric acid, and crystallization of the salts formed, was also unsuccessful. Transformation of **26** into oxazolidone **27**, by the reaction with diethyl carbonate [23], gives oxazolidone **27** in only 30% yield. Fortunately, its yield increased to 75% when triphosgene was used instead of diethyl carbonate. Finally, crystallization of **27** followed by alkaline hydrolysis gave **26** of high purity, as indicated by ^{1}H, ^{13}C NMR, and HPLC analyses [20].



Scheme 3

Debenzylation of (*R*)-**15** was the next step in the synthesis of **1** (Scheme 4), however, attempts to achieve it by catalytic hydrogenolysis on Pd/C or $Pd(OH)_2$/C resulted in the nitrogen – oxygen bond cleavage. Treatment of (*R*)-**15** with boron trichloride gave a mixture of products, instead of the expected debenzylation. Consequently, (*R*)-**15** was transformed into the corresponding *N*-benzyloxyurea derivative **28** which was readily debenzylated by palladium catalyzed hydrogenolysis producing **1**, 92% ee, and after upgradation by crystallization, 99% ee was obtained [20].

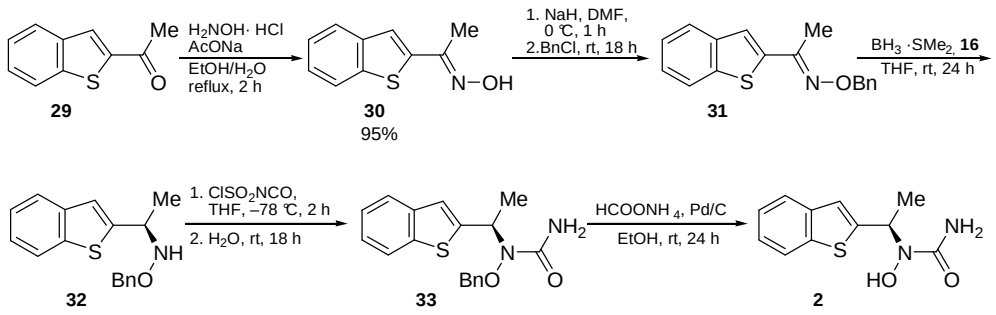


28 95%

1 83%, 99% ee

Scheme 4

The same approach, via the reduction of oxime ether, was applied to the asymmetric synthesis of **2**, starting from 1-(benzo[*b*]thiophen-2-yl)ethanone **29** (Scheme 5). The ketone was converted into oxime **30**, and its *O*-benzyl ether **31** which was reduced. The reduction with borane/oxazaborolidine, generated from (1*S*,2*R*) norephedrine, gave the corresponding hydroxylamine oxime ether **32**, 80% ee, in 49% yield. In the presence of oxazaborolidine **22** the enantioselectivity of the reduction increased to 95%. The product hydroxylamine *O*-benzyl ether **32** was transformed into the corresponding *N*-benzyloxyurea **33** by the reaction with chlorosulfonyl isocyanate. In contrast to debenzylation of the benzofuran analogue **28**, (Scheme 4) hydrogenolysis of **33** in the presence Pd/C or $Pd(OH)_2$/C was very sluggish and incomplete even after prolonged periods. Debenzylation was achieved with ammonium formate in the presence of Pd/C in ethanol, as reported earlier in the synthesis of **2** by a different approach,[9] however, a substantial load of the catalyst was necessary [20].



29

30 95%

31

32 50%, 95% ee

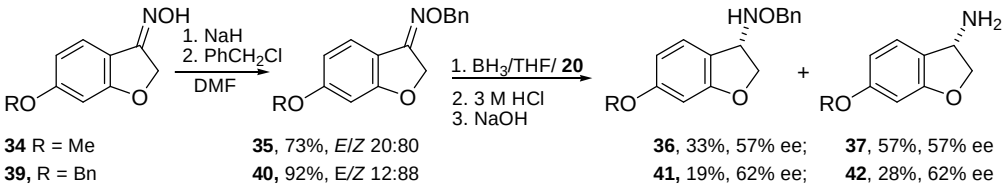
33 89%

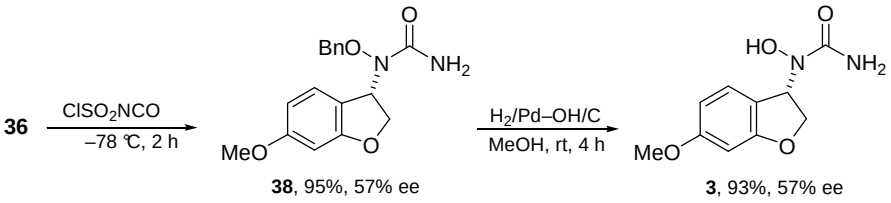
2 70%, 95% ee

Scheme 5

Next, we turned to the synthesis of *N*-(6-methoxy2,3-dihydro-benzofuran-3-yl)-*N*-hydroxyurea **3**. In the first approach, the synthesis of (*Z*)-6-methoxy-2,3-dihydrobenzofuran-3-*P,P*-diphenylphosphinylimine was under-taken following the procedure reported for substituted acetophenones [24]. However, 6-methoxybenzofuran-3-*P,P*-diphenylphosphinylimine was obtained, instead of the expected product.

In the second approach, 6-methoxy-2,3-dihydrobenzofuran-3-one oxime *O*-benzyl ether **35,** *E/Z* 20 : 80, was prepared (Scheme 6). The ether was reduced with borane/oxazaborolidine **20** to give **36,** 57% ee, and amine **37**, 57% ee. Enantioselectivity of the reduction is influenced by the *E/Z* ratio, and increased to 84% ee when the ratio was 8:92. The **36** hydrochloride, crystallizing during the work-up of the reduction products, was separated from the soluble **37** hydrochloride. Free **36** was liberated by alkalization, and was converted into the corresponding *N*-benzyloxyurea **38**. The debenzylation of **38** by hydrogenolysis on the Pd/C catalyst was readily achieved, and *N*-hydroxyurea **3**, 57% ee, was obtained [25].

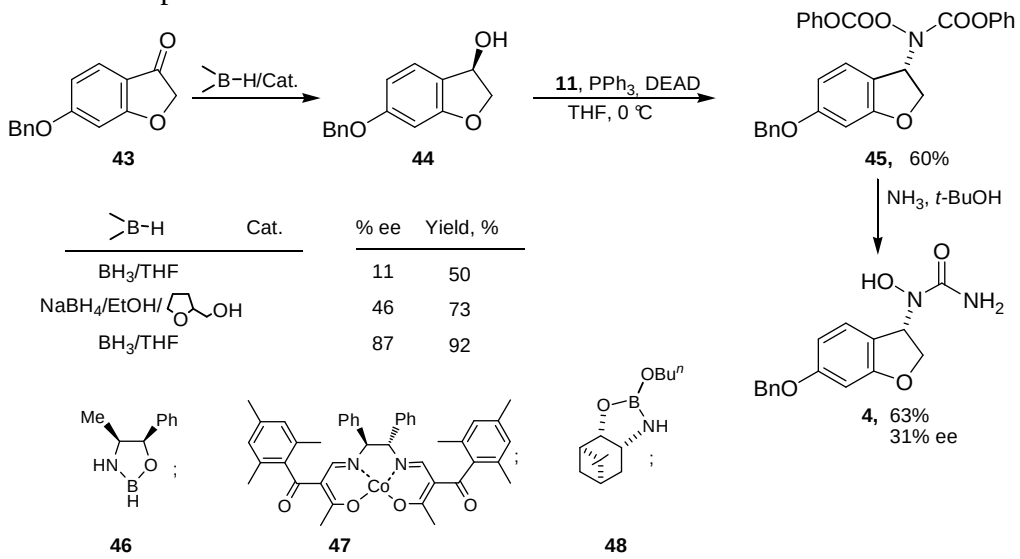




Scheme 6

The asymmetric synthesis of *N*-6-benzyloxy-2,3-dihydrobenzofuran-3-yl)-*N*-hydroxyurea **4** was the most challenging. Following the same approach as described above for the 6-methoxy analogue **3,** the 6-benzyloxy group may interfere in debenzylation of **41** (Scheme 6). In order to avoid this inconvenience, in the first approach, 6-benzyloxy-2,3-dihydroxybenzofuran-2-on **43** was reduced to the corresponding alcohol **44** (Scheme 7). Reductions with borane/oxazaborolidine **46**, generated from (1*R*,2*S*)-norephedrine, and with modified sodium tetrahydroborate in the presence of β-ketoimina-tocobalt(II) complex [26] **47**, gave **44** in moderate enantiomeric excess.

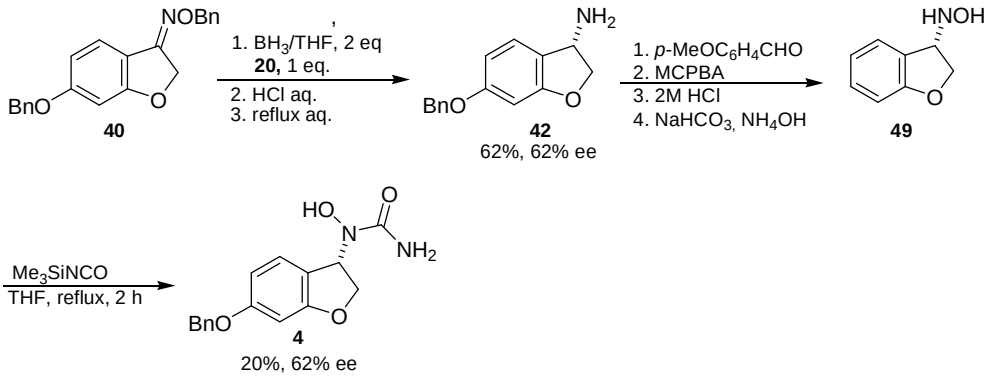
Fortunately, the reduction with borane/oxazaborolidine **48**, generated from (1*R*,2*S*,3*R*,5*S*)-3-amino-6,6-dimethylbicyclo[3.1.1]heptan-2-ol [27], derived from (–)-β-pinene, was more selective producing **44** in 87% ee. However, treatment of the alcohol with *N*,*O*-bis(diphenoxycarbonyl)hydroxylamine **11** under Mitsunobu conditions, followed with ammonia, gave partially racemized *N*-hydroxyurea **4**, 31% ee. Clearly, 6-benzyloxy-2,3-dihydrobenzofuran-3-yl group influences the substitution reaction in a similar way to 1-(*o*-methoxy- and 1-(*p*-methoxyphenyl)ethyl group [21,22]. It should be noted that under the same conditions (*S*)-1-phenylethanol, 93% ee, reacted with **11** to give the substitution product in 88% ee.



Scheme 7

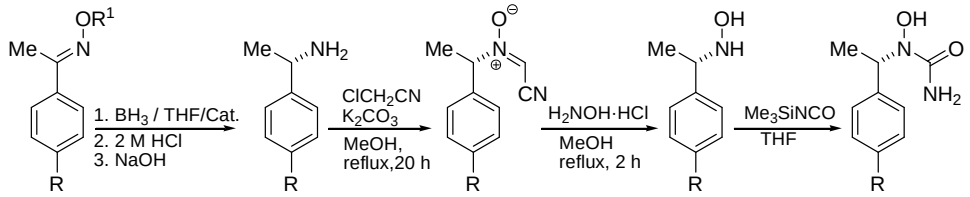
In the second approach, 6-benzyloxy-2,3-dihydroxybenzofuran-3-one **43** was transformed into its oxime *O*-benzyl ether **40** (Scheme 6). The ether was earlier reduced with borane/oxazaborolidines prepared from various β-amino alcohols, and only in the presence oxazaborolidine **20**, generated from (1*S*,2*R*)-norephedrine, the reduction product was obtained in high enantiomeric excess [6]. In our experiments, *O*-benzyl ether **40** *E*/*Z* 12:88, reduced with borane/oxazaborolidine **20** gave a mixture of the corresponding hydroxylamine *O*-benzyl ether **41**, 62% ee, 19% yield, and 3-amino-6-benzyloxy-2,3-dihydrobenzofuran amine **42**, 62% ee, 28% yield (Scheme 6). The ether **42** was separated and converted into the corresponding *N*-benzyloxy-*N*-hydroxyurea in the way described above for the 6-methoxy analogue. However, debenzylation by catalytic hydrogenolysis resulted mainly in the removal of both benzyl groups, and (*S*)-*N*-(6-hydroxy-2,3-dihydrobenzofuran-3-yl)amine was obtained as the major product.

Consequently, the approach was modified and *O*-benzyl ether **40** was reduced with an excess of borane/oxazaborolidine **20** to amine **42** obtained in 62% yield, (Scheme 8). A few methods for the oxidation of amines to hydroxylamines are known [28-38]. We tested the most convenient ones, and only one transformation, via oxazirine [30], produced the desired hydroxylamine **49** which was transformed into **4** by treatment with trimethylsilyl isocyanate [25].



Scheme 8

The approach to *N*-hydroxyureas via amines is a general methodology, and was also used in the asymmetric synthesis of *N*-hydroxyureas derived from substituted acetophenones. (Scheme 9). Thus, acetophenone was transformed into its (*E*)-oxime *O*-benzyl ether, and *p*-methoxy and *p*-benzyloxyacetophenones were transformed into their (*E*)-oxime *O*-2-nitrobenzyl ethers. The reduction of **50** with borane/oxazaborolidine **46**, generated from (1*R*,2*S*)-norephedrine, and the reduction of **52** and **54** with borane/oxazaborolidine **55**, generated from (*S*)-diphenylvalinol, produced cleanly the corresponding amines **51**, **53**, **55**, 99, 98 and 93% ee, in 85, 79, and 67% yield, respectively. The amines were transformed, via nitrone [29], into the corresponding *N*-hydroxyureas **5–7,** with no racemization. This sequence of transformations: primary amine – *N*-cyanomethyl amine – nitrone – N-substituted hydroxylamine, followed by its conversion into N-substituted *N*-hydroxyurea, worked well for these amines.



R	R^1	Cat.		
50, H	Bn	**46**	**51,** 85%, 99% ee	**5,** 63%, 99% ee
52, MeO	*2*-NO_2Bn	**56**	**53,** 79%, 98% ee	**6,** 64%, 98% ee
54, BnO	2-NO_2Bn	**56**	**55,** 67%, 93% ee	**7,** 56%, 93% ee

56 = oxazaborolidine prepared from (*S*)-(–)-diphenylvalinol

Scheme 9

Conclusions

New highly selective terpene oxazaborolidines have been used. The enantioselective reduction of prochiral ketoxime *O*-ethers with borane/ oxazaborolidines can be controlled to give the corresponding hydroxylamine *O*-ethers or amines. The hydroxylamine *O*-ethers can be converted into the corresponding *N*-hydroxyureas, provided that the deprotection of the ether functionality can be achieved. Alternatively, the amines can be transformed into the corresponding *N*-hydroxyureas avoiding the above mentioned limitation. The methodology described above is applicable to the asymmetric synthesis of similar structures.

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